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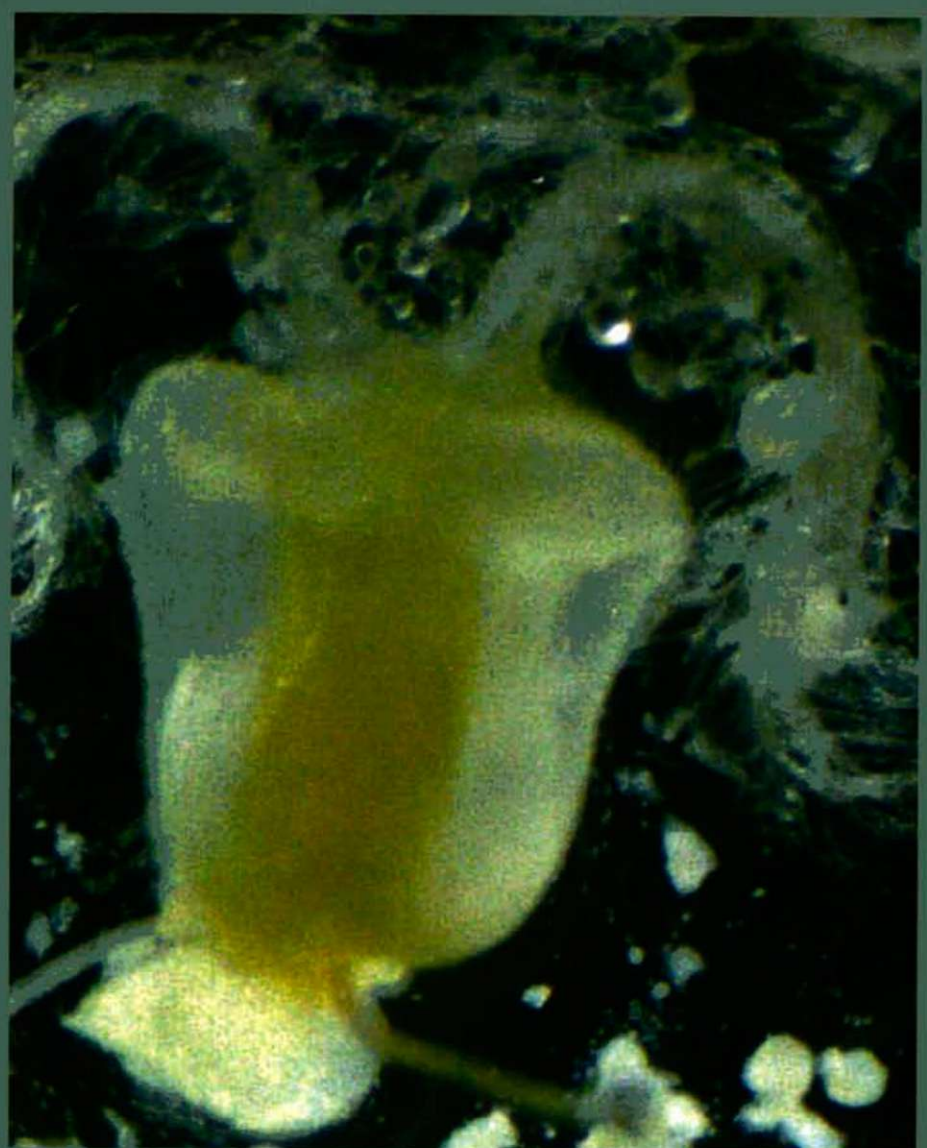

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REVIEW ARTICLE

Plant Gene Regulatory Network System Under Abiotic Stress

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ABSTRACT Plants differ from animals in many aspects, but the important may be that plants are more easily influenced by environment than animals. Plants have a series of fine mechanisms for responding to environmental changes, which has been established during their long-period evolution and artificial domestication. These mechanisms are involved in many aspects of anatomy, physiology, biochemistry, genetics, development, evolution and molecular biology, in which the adaptive machinery related to molecular biology is the most important. The elucidation of it will extremely and purposefully promote the sustainable utilization of plant resources and make the best use of its current potential under different scales. This molecular mechanism at least include environmental signal recognition (input), signal transduction (cascades of biochemical reactions are involved in this process), signal output, signal responses and phenotype realization, which is a multi-dimensional network system and contain many levels of gene expression and regulation. We will focus on the molecular adaptive machinery of plants under abiotic stresses and draw a possible blueprint for it. Meanwhile, the issues and perspectives are also discussed.

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KEY WORDS

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Human being has stepped into the 21th century, during which sustainable - healthy utilization for environment and resources and its own health concerns are the most important issues (Rosenberg et al. 1993). These issues are tightly linked with agriculture (food) and eco-environment, in which biology, in particular plant biology plays the greatest role, because plants offer the globe its only renewable resource of food, building material and energy and thus plant biology is the most powerful tool to reasonably use natural resources (Bazzaz 2001; Charlesworth et al. 2001; Agraval et al. 2003; Anand et al. 2003; Chaves et al. 2003; Munns 2003, 2005; Shao et al. 2003, 2005; Angela 2004; Arnholdt-Schmitt 2004; Brian et al. 2004). In the broad field of plant biology, its core is the study for life activities at molecular level (mainly DNA and protein macromolecules), whose interactions at various biointerfaces at different scales are quite important to keep a steady state between plants and changing environment, especially adverse surroundings (Doebley and Lukens 1998; Avramova 2002; Kreps et al. 2002; Ambros et al. 2003; Chaves et al. 2003, 2004; Shao et al. 2003, 2004, 2005a-d; Beer and Tavazoie 2004; Casati and Walbot 2004; Chinnusamy et al.2004; European Commission 2004; Jiang and Zhang 2004; Ashraf and Chu et al. 2005; Foolad 2006. So, adaptation in plants is an important and timely topic in basic and applied biology (Bonnie et al. 1998; Eckardt et al. 2001; Dufty et al. 2002; Brill and Watson 2004; Castle et al. 2004; David et al. 2004; Editor's choice 2004; Hiral et al.2004; Ma 2004; Shao et al. 2004; Andrew et al. 2006; Humphreys et al. 2006). On the one hand, it is very interesting to understand interaction between plants and their environment. On the other hand and in view of the needs for human life, we more want to create crop plants that are able to confront successfully unfavorable natural conditions (Fischer et al. 2000; Chen et al. 2002; Doelle 2002; Brill and Watson 2004; Capell et al. 2004; Casu et al. 2004; Costa et al. 2004; Delessert et al. 2004; De Ronde et al. 2004). The main aim in plant breeding is to obtain plants that combine higher yields, reliable yield stability, better quality and obvious characters resisting stresses (abiotic and biotic) over years and locations (Liu et al. 2000; Chen et al. 2002; Fiehn 2002; Gesch et al. 2002; Graves and Haystead 2002; Castle et al. 2004; Chen and Gal-

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lie 2004; Glombitza et al. 2004; Hao et al. 2004; Lu and Chen 2004; Liu and Li 2005; Grennan 2006). However, in addition to biotic stress factors, disturbances of extreme or even mild abiotic stress are supposed to account for a high amount of unachieved potential in plant production all over the globe (Kasuga et al. 1999; Harmer et al. 2000; Johson et al. 2001; Halford and Paul 2003; Lokhandle et al. 2003; Shinozaki et al. 2003; Gregory et al. 2004; Harding et al. 2004; Kennedy and Wilson 2004; Liu et al. 2004; Mark and Antony 2005; Shao et al. 2006d). Diverse forms of abiotic stresses may occur, including drought, cold and freezing, heat, salinity, nutrient deficiency, toxic heavy metals, oxidative stress as well as oxygen shortage, and mechanical stress (Mlot 1998; Harmer et al. 2000; Kwon and Kim 2001; Meyerowitz 2002; Halford and Paul 2003; Miyao 2003; Hernandez et al. 2004; Higuchi et al. 2004; Kim et al. 2004; Liu and Baird 2004; Medict et al. 2004; Munne-Bosch and Alegre 2004; Bartha et al. 2005; Kolbert et al. 2005; Grennan 2006; Liu and Bush 2006). Although it is accepted that diverse environmental stress factors never act alone, experimental study of plant responses to abiotic stress is normally restricted to plant reactions on isolated stress factors (Neumann 1997; Riechmann et al. 2000; Somerville and Dangl 2000; Mette et al. 2002; Noctor et al. 2002; Pellegrineschi et al. 2002; Travis et al. 2002; Millar et al. 2003; Tang et al. 2003; Wang et al. 2003; Manival et al. 2004; Puhakainen et al. 2004; Rae et al. 2004; Soltani et al. 2004; Taylor et al. 2004; Philippe et al. 2005; Shao et al. 2005, 2005a-e, 2006d; Sun et al. 2005; Tan et al. 2006). However, it has to be considered that stress always occurs as a complex of various interacting environmental factors that contribute in varying degrees to the overall stressed phenotype (Zhou et al. 2000; Vranova et al. 2002; Yong et al. 2003; Salt 2004; Wang et al. 2004; Wei et al. 2004; Winichayakul et al. 2004; Yang et al. 2006; Yang and Zhang 2006). Consequently, plants usually respond to a unique complex of growth conditions (Zhu 2002; Zhu 2003; Zhu T 2003; Zhu et al. 2004; Shao et al. 2006). Stress inducers from the abiotic as well as biotic world have some common signal and responding pathways in plants (Samis et al. 2002; Schlighting 2002; Vranova et al. 2002; Shinozaki and Dennis 2003; Tang et al. 2003; Shao and Chu 2005; Shao et al. 2006b,f) and thereby have the potential to moderate the effect of each other through cross-talking (Riechmann et al. 2000; Samis et al. 2002; Shigeoka et al. 2002; Soltis and Soltis 2003). Further, plants, as sessile organisms, have to get alone with the dynamics of transiently changing environmental conditions and have the flexibility for responding to these complicated changes (Manival et al. 2001; Poethig 2001; Pellegrineschi et al. 2002; Munns 2003, 2005; Rae et al. 2004), and this has to be achieved at the various stages of plant development (Kreps et al. 2002; Meyerowitz 2002; Hiral et al. 2004; Kim et al. 2004; Mark and Antony 2005; Shao et al. 2005d; Liu and Bush 2006).

Considering the interacting complexity (at least including water movement, solute transport, information exchange, ion homeostasis regulation, and other related physico-chemical changes) between plants and their surroundings, it is necessary to generalize first the performance of physiological functions for plants under soil water stress in this article. We then focus on the aspects of plant gene regulatory network system, which is the core controlling the interrelationship between plants and environment at the molecular level in a complex and coordinated manner. Drought will be selected as an example of abiotic stresses to illustrate the above issue.

Plant physiological function performance under soil water stress

Plants live in soil-plant-atmosphere continuum (SPAC) environment, and they have to coordinate the mechanisms of diverse types to respond to the above changing environment at any time for sustainable survival (Dufty et al. 2002; Fiehn 2002; Glombitza et al. 2004; Gregory et al. 2004; Shao et al. 2005). Plant production realization is obtained eventually through physiological pathways at least at the level of individual and community (Charlesworth et al. 2001; Chaves et al. 2003; Angela 2004; Arnholdt-Schmitt 2004; Brill and Watson 2004; Capell et al. 2004; Chen and Gallie 2004; Shao et al. 2005, 2006a,c; Andrew et al. 2006). One molecule, one kind of tissue or an organ can not produce any economic yield in terms of the need for human being (Soltani et al. 2004; Munns 2005; Shao et al. 2005, 2006c).Under the condition of ensuring plant survival, plants can produce corresponding yield. Water is one of key factors influencing plant production and many reports have proved this clearly (Travis et al. 2002; Salt 2004; Yang and Zhang 2006). Loss of water in soil will lead to great reduction in plant production, which has been reflected from total grain yield of many countries in the world (Doelle 2002; Wang et al. 2003; Shao et al. 2006c,e). Water is the important material for photosynthetic reactions that plants depend on to finish accumulation of photosynthetic products, which are impacted greatly by physiological pathways and environmental factors (such as soil water supply; Angela 2004; David et al. 2004) Thus, different soil water supplying will result in quite different physiological pathways, which directly determine the ability for plants to make photosynthetic products. Water deficits in soil environment also influence solute transport (ion and nutrient uptake of plants) to larger extent, which effects on photosynthetic reactions in plant chloroplasts in many ways (Salama et al. 1994; Zhu 2002; Halford and Paul 2003; Lokhandle et al. 2003; Chaves and Oliveira 2004; Costa et al. 2004; Higuchi et al. 2004; Salt 2004; Andrew et al. 2006). This is the reason that ion homeostasis and redox state have been brought to attention (Noctor et al. 2002; Samis et al. 2002; Shigeoka et al. 2002; Vranova et al. 2002; Millar et al. 2003; Harding et al. 2004; Taylor and McAinsh 2004; Grennan 2006). The series of the above reactions and processes occurring at

different biointerfaces is regulated and controlled by plant gene regulatory network system spatially and temporally on the basis of responding to plant developmental cue, through which plants can elegantly respond to the changing environment (Hernandez et al. 2004; Grennan 2006). This network system has been formed by the interaction between plants and environment for a long time of evolution, which will continue to evolve with environmental succession (Dufty et al. 2002; Gesch et al. 2002; Charlesworth et al. 2004; David et al 2004; De Ronde et al. 2004). From the angle of individual plant development, Plant Growth Periodicity curve can reflect and show the above trend (Doebley and Lukens 1998; Soltani et al. 2004; Shao et al. 2005c,e). Besides, plant responses to soil water deficits take a "slow-fast-slow" shaped curve in terms of main physio-biochemical indices change and this is in agreement with Plant Growth Periodicity, which also illustrates this fact and wide plasticity for plants (Neumann 1997; Poethig 2001; Schlighting 2002; Shao et al. 2005a, 2006a). Surely, concerted expression of corresponding genes in plant gene regulatory network system makes it possible that we can see the phenotype and phenotype change under given temporal-spatial condition (Shinozaki et al. 2003; Shinozaki and Dennis 2003; Zhu 2003; Taylor and McAinsh 2004; Shao et al. 2006d).

Aspects of plant gene regulatory network system

Recent progress in molecular biology (especially, DNA microarray), genomics, proteomics and metabolomics has provided insight into plant gene regulatory network system, which is mainly composed of inducible-genes (environmental factors and developmental cues), their expression programming and regulatory elements (cis-element and trans-element), corresponding biochemical pathways and diverse signal factors (Tang et al. 2003; Wang et al. 2003; Zhu 2003; Zhu T 2003; Munns 2005). Under the condition of soil water deficits, related stress factors always result in overlapping responses, including anatomical, physiological, biochemical, molecular biological changes, which make plant gene regulatory network system more complicated and difficult to explore. Much information with respect to this topic is from the model plant, *Arabidopsis thaliana*. Main aspects will be illustrated below.

Environmental stress-responsive transcriptional elements

Plants can sense, process, respond to environmental stress and activate related-gene expression to increase their resistance to stress. Environmental stress-inducible genes can be mainly divided into two types in terms of their protein products: one type of genes, whose coding products directly confer the function of plant cells to resist to environmental stress such as LEA protein, anti-freezing protein, osmotic regulatory protein, enzymes for synthesizing betaine, proline and other osmoregulators; the other type of genes, whose coding products play an important role in regulating gene expression and signal transduction such as the transcriptional elements for sensing and transducing the protein kinases of MAP and CDP, bZIP, MYB and others (Liu et al. 2000; Szegletes et al. 2000; Liu et al. 2004; Lu and Chen 2004; Liu and Bush 2006). Transcriptional elements are defined as the protein combining with the specialized DNA sequence of eukaryotic promoters or the protein having structural characteristics of known DNA-combining region, whose main function is to activate or suppress transcriptional effect of corresponding genes (Kasuga et al. 1999; Liu et al. 2000; Manival et al. 2001; Mette et al. 2002; Gregory et al. 2004; Liu and Baird 2004; Shao et al. 2005). Up to now, hundreds of transcriptional elements of environmental stress-responsive genes in higher plants have been isolated, which regulate and control the stress reaction related to drought, salinity, cold, pathogen and heat (Doebley and Lukens 1998; Tang et al. 2003; Delessert et al. 2004; Glombitza et al. 2004). In the genome of *Arabidopsis* and rice, they have about 1300-1500 genes for coding transcriptional elements, most of which have not been identified functionally. Recent study has shown that the transcriptional elements involved in plant stress responses mainly include four kinds: APETALA2/EREBP, bZIP, WRKY, and MYB. Typical transcriptional elements have been summarized in Table1 for reference.

Table 1. Typical transcriptional elements in higher plants.

Plant materials	Factors	Binding sites/Factor Types
Arabidopsis thaliana	ABI5/AtDPBF	ABA response elements(ABREs)/bZIP
A.thaliana	AtDPBF2	ABA response elements(ABREs)/bZIP
A.thaliana	AtDPBF3/AREB3	ABA response elements(ABREs)/bZIP
A.thaliana	AtDPBF4	ABA response elements(ABREs)/bZIP
A.thaliana	AtDPBF5/ABF3	ABA response elements(ABREs)/bZIP
A.thaliana	ABF1	ABA response elements(ABREs)/bZIP
A.thaliana	ABF2/AREB5	ABA response elements(ABREs)/bZIP
A.thaliana	ABF4/AREB2	ABA response elements(ABREs)/bZIP
A.thaliana	GBF3	ABA response elements(ABREs)/bZIP
A.thaliana	AB53	RY/sph elements/B3 domain proteins
A.thaliana	ATMTB2	MTC
A.thaliana	ATHB6	HD-Zip
A.thaliana	ATHB7	HD-Zip
A.thaliana	ATHB12	HD-Zip
A.thaliana	ABI4	AP2
Oryza	TRAB1	ABA response elements(ABREs)/bZIP
Oryza	OsVPI	RY/sph elements/B3 domain proteins
Zea mays	VP1	MYB
Triticum	EmBP-1	ABA response elements(ABREs)/bZIP
Avena	AtVPI	RY/sph elements/B3 domain proteins
Helianthus	DPBF5,-2,-3	ABA response elements(ABREs)/Bzip
Phaseolus	ROM2(repressor)	ABA response elements(ABREs)/Bzip
Phaseolus	PIARF	RY/sph elements/B3 domain proteins
Craterestinma	Cpvp1	RY/sph elements/B3 domain proteins
Daucus	C-ABI3	RY/sph elements/B3 domain proteins
Populus	PtABI3	RY/sph elements/B3 domain proteins

Complexity of plant gene regulatory network system specificity and crosstalk

Many transcriptional element families participate in plant stress responses, each of which has many members with highly-conservative DNA-binding domain, composing a complicated, temporal-spatial network system for plant gene expression and regulation (Zhu 2003; Zhu T 2003). Different members of TGA/OBF families have different DNA-binding specificities, protein-protein interaction and expressing profiles. Chromatin immunoprecipitation techniques indicated that tobacco TGA1a *in vivo* combined with xenobiotic-responsive promoters, but could not combine with PR promoter as cis-element (Beer and Tavazoie 2004; Chinnusamy et al. 2004). *Arabidopsis* TGA2 could be responsive to salicylic acid (SA) signal, but not be responsive to xenobiotic stress signals. Much analysis of genomic expression profiling by DNA microarray indicates that the mRNA coding transcriptional element genes in many plants are usually induced to express and accumulated (Avramova 2002; Arnholdt-Schmitt 2004; Casati and Walbot 2004). Most transcriptional element genes involved in plant stress responses have not only completely different expression profiles, but also some overlapping expression profiles, showing the complexity, specificity and crosstalk of plant gene regulatory network system (Bray 2004; Shao et al. 2005, 2006d). In other words, one kind of stress may simultaneously activate many transcriptional elements and one transcriptional element may be activated by many types of plant stress responses. For instance, CBF3/DREB1a can be responsive rapidly to cold, at the same time, regulated by circadian clock (Harmer et al. 2000; Chen et al. 2002; Brill and Watson 2004), which reflects the functional complement between plant cold-responsive pathway and circadian clock-regulated circle in terms of CBF3/DREB1a functions.

Shinozaki et al. (2003) thought that four signal pathways were involved in plant drought, cold and salinity responses, in which two were ABA-dependent (I and II), and two were non-ABA-dependent (III and IV). The process of stress signal sensing and transducing, transcriptional regulating, and functional expressing was existent in these pathways. It is obvious that transcriptional elements play a central role in the process (Liu et al. 2000; Shao et al. 2005, 2006d). Zhu T (2003) and Zhu JK (2003) concluded that molecular mechanism of plant stress responses to drought and salinity included three main steps, *i.e.* stress signal input, transducing process, and regulatory product output through the study of *Arabidopsis* drought and salinity for many years. Results of many genetic mutants and key intermediate molecules from his lab supported his view powerfully. Recent related anti-drought data (dynamic change of anti-oxidative enzymes and soil water stress threshold) from my lab also proved the point (Shao et al. 2005a-e, 2006b,e). From plant developmental context, plant responses to environmental stresses have a universal law, which has been reflected completely by Plant Growth Periodicity curve (Shao et al. 2005c). Our study on dynamic changing of wheat anti-oxidative enzymes under soil water deficit have indicated that wheat with different genotypes responded to soil water stress by taking a "slow-rapid-slow" characteristic curve during wheat life cycle (Shao et al. 2005, 2006b).This is the physiological basis for water-saving agriculture and dry land farming,which also provides substantial evidence for the above viewpoint (Chaves and Oliveira 2004; Munne-Bosch and Alegre 2004; Shao et al. 2006c).

Plant gene regulatory network system and plant drought resistance improvement

Previous gene engineering strategy for plant stress resistance was to express one (in most cases) or several stress-tolerant genes by constitutive or stress-induced promoters (Pellegrineschi et al. 2002). For instance by introducing betA gene derived from *E.coli* into tobacco and potato, betaine content in the transgenic plants increased to 5 μmol/g (dry mass) and tolerance to salt and cold for the transgenic plants was improved greatly. The goal of recently-established plant gene engineering strategy based on transcriptional elements is to improve plant comprehensive resistance characters (Puhakainen et al. 2004). Compared with the previous traditional method of introducing or improving individual functional genes, the new strategy will play more important role in plant molecular breeding because modifying regulatory activities of a transcriptional element can influence functions of many genes, easily reaching the aim of improving plant comprehensive resistance to drought, salinity, freezing, diseases, UV-B and others (Riechmann et al. 2000; Capell et al. 2004; Casati and Walbot 2004). Through constitutively overexpressing DERB1A, plant stress-responsive genes, Kin1, cor6.6/Kin2, cor15a, cor47/rd17, or d10 got higher

Table 2. Some examples of the osmotic regulating genes downstream in abiotic resistance.

Components	Metabolic Functions	Gene/Proteins
ROS scavenging	Increase in ROS scavenging enzymes	GP, PH, GPX
Chaperones	Heat-/cold-/salt-shock proteins; protein folding	Hsp,Csp,Ssp,*DnaJ*
Fructan	Osmoprotection	*SacB*
Trehalose	Osmoprotection	*Tps;Tpp*,trehalase
Glycine betaine	Protein protection and carbon sink	*codA*
Proline	Substrate for mitochondrial respiration; redox control	P5CS/P5CR
Ectoine	Osmoprotectant	*Ect*A,BC
K^+-transporters	High affinity K^+ uptake	Hkt1,Hak1
K^+-channels	Low affinity or dual affinity K^+ uptake	Akt1,Akt
H_2O channel proteins	Membrane cycling control	TIP

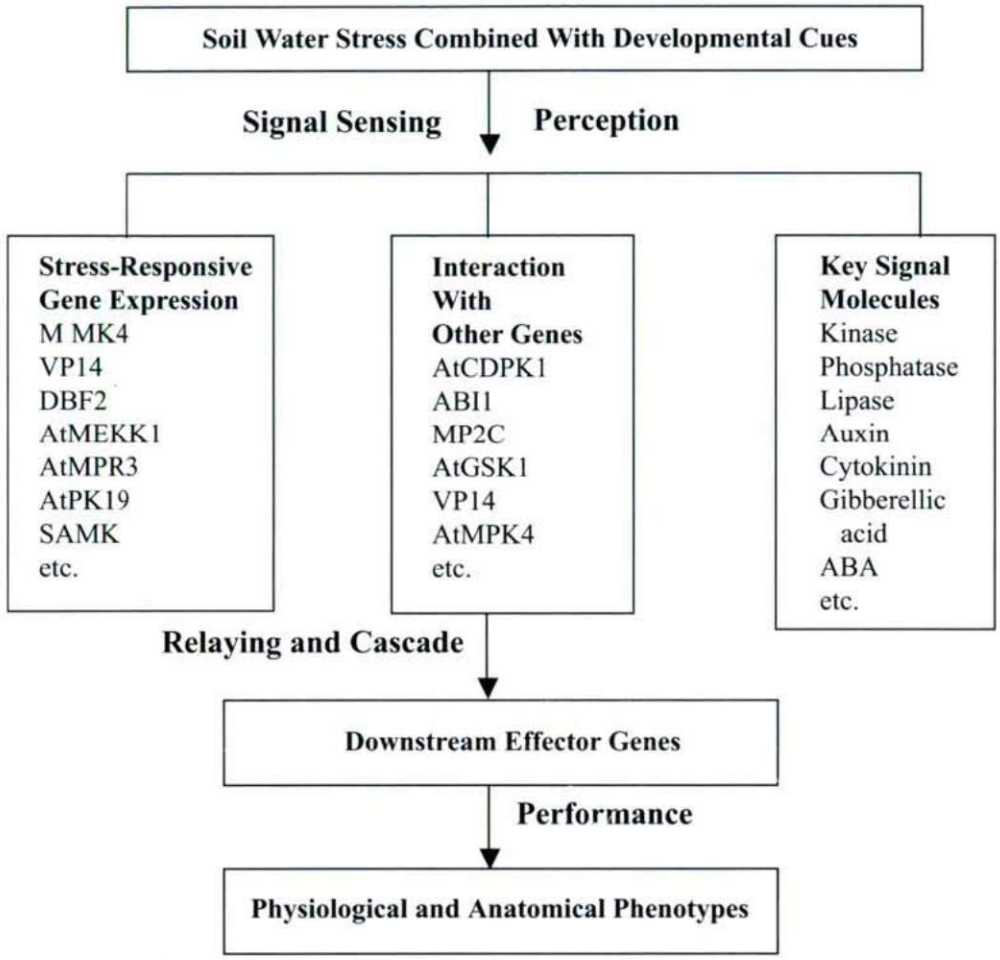

Figure 1. The basic draft for plant gene regulatory network system.

expression and the obtained transgenic *Arabidopsis* plants were resistant to drought, cold and salinity (Kasuga et al. 1999; Pellegrineschi et al. 2002, Puhakainen et al. 2004). Other related studies also provided a solid evidence for high efficiency of the above methodology.

Transferring a transcriptional element into *Arabidopsis*, which was thought previously not to be related to plant drought response many transgenic plants were obtained which were highly resistant to soil water deficits on the basis of selecting the *Arabidopsis* community with higher expression. By further introducing the members of this transcriptional element family into soybean, transgenic soybean lines were cultured and they were resistant to soil water deficits in greenhouse and field. This indicated that the function of this transcriptional element family was characteristic of conservativeness among diverse plant species. So, it is possible to obtain expected same stress-resistant phenotype by genetically modifying transcriptional elements and reach the aim of improving plants efficiently and purposefully (Tang et al. 2003; Wang et al. 2003; Zhu et al. 2004; Shao et al. 2006d). Besides, some transcriptional elements not only regulate metabolic pathways, but also influence transport and allocation of secondary metabolites. Plant secondary metabolism plays an important role in plant responding to environmental stresses. Long-step progress has taken place in terms of introducing transcriptional elements to regulate targeted pathways.

It is important to remember the fact that some transcriptional elements may regulate several metabolic pathways and one metabolic pathway may need orchestrated regulation from some transcriptional elements, which is the nature of plant gene regulatory network system (Shinozaki et al. 2003; Zhu 2003; Zhu T 2003; Shao et al. 2006d). So, in some cases, only introducing a transcriptional element can not obtain targeted phenotype and may lead to metabolic unbalance in plants. In addition, because of coordinated evolution of transcriptional elements and their regulating metabolic pathways the genetically-modifying strategy for the same transcriptional element

could produce different phenotypes in different plant species. These issues need deeper exploration to establish an efficient genetically-modifying system by transcriptional elements and their network system for improving plant stress resistance and global eco-environment and feeding the world (Shao et al. 2006c).

Concluding remarks

Between plants and animals the most important difference is that plants are more easily influenced by environmental factors than animals. Consequently, plants have more refine mechanisms to regulate themselves from molecular level to ecosystem to respond to environmental changing. For instance, there are many coding-protein genes downstream only for osmotic regulation in abiotic stress resistance (Table 2). By contrast, animals are more active and have the ability to escape from environmental stresses in most cases (Meyerowitz 2002; Wei et al. 2004).Under the above background, plants are quite different compared to animals in their gene regulatory network system (Wei et al. 2004). Nerve system-based or nerve system-like-based structure and hormones are composed of the body for gene expression in animal network system, leading to animal activeness (Meyerowitz 2002; Shao et al. 2006d). In addition, developmental programming can not be easily effected by environmental cues (Munns 2005; Shao et al. 2005). Plants are always in the state of passiveness for confronting environmental succession and the related issue is more complicated, which is the main cause that plants are behind animals in the study of most fields (Shao et al. 2006d).

Charting plant gene regulatory network system under soil water deficits is a great challenge. Nowadays, there are indeed many favorable conditions for charting this blueprint, including much available data from *Arabidopsis*, rice, grass, yeast and fruit fly, but the range of tested plants is very much limited, many stress-responsive genes have not been unified in terms of their refine functions, and many genes participating in environmental stresses are interacted and overlapped, which have led to incorrect placing of key genes (gene effectors) and signal molecules in the whole plant gene regulatory network system. Besides, much data are from under condition of one type of stresses. It is a fact that plants always confront more than two kinds of individual environmental stresses or their combination simultaneously in field (Soltani et al. 2004; Liu and Li 2005; Shao et al. 2005; Andrew et al. 2006; Yang et al. 2006; Yang and Zhang 2006). Although drawing this dimensional plant gene regulatory network system with great details and complete pathways is impossible currently, the basic draft for this blueprint could be summarized in Figure 1. This draft was established in combination with recent advance in this hot topic and from the context of development, which will provide instructions for further investigation and insights into understanding of plant refine plasticity for abiotic environmental stresses.

In a word, precise elucidation of plant gene regulatory network system under abiotic stresses is of importance to molecularly engineering plant resistance, because of which many excellent scientists world-wide have been engaged in this frontier field, resulting in a long-step progress (Shao et al. 2005, 2005b; Shao and Chu 2005).There are also many issues remained to be solved and needed to make efforts. Scope of tested plants needs to be extended; comprehensive study on a combination of environmental stress factors in laboratories and in field should be given much attention; system development viewpoint and computer simulation analysis method should be also applied. With accumulation of data from being extended plant range, plant gene regulatory network system under environmental stresses will be clearer and clearer.

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ARTICLE

Effects of drought on photosynthetic parameters and heat stability of PSII in wheat and in *Aegilops* species originating from dry habitats

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ABSTRACT The effects of water deficit induced by withholding water in soil pots were examined on processes related to photosynthesis and heat stability of PSII in wheat cultivars and in *Aegilops* species. Decrease in relative water content (RWC) of leaves resulted in fast and considerable stomatal closure and decrease in net photosynthetic CO_2 fixation (A) in *Ae. bicornis* and in wheat cultivars, while in *Ae. tauschii* and *Ae. speltoides* stomatal conductance (g_s) and A remained relatively high between 90 and 70% RWC. Parallel with this, A was limited by the CO_2 diffusion to the intercellular spaces (stomatal limitation, L_s) even at a lower RWC in *Ae. taushii* and in *Ae. speltoides*, while a significant mesophyll limitation (L_m) was observed for *Ae. bicornis* and for wheat. On the other hand, drought stress resulted in a significant increase in the thermal stability of PSII in wheat and *Aegilops* genotypes. The results indicate that some genotypes of *Ae. taushii* and *Ae. speltoides* have better drought tolerance with satisfactory heat stability than wheat, making them appropriate for improving the heat tolerance of wheat to survive dry and hot periods in the field.

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KEY WORDS

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stomatal conductance
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Aegilops species with good tolerance to some major abiotic stress factors are closely related to wheat (Van Slageren 1994) and widely used as genetic resources for *Triticum* species (Molnár et al. 2004). As reported, the tetraploid goat grass (*Aegilops biuncialis* L., 2n = 4x = 28, $U^bU^bM^bM^b$) has a good drought tolerance, which makes it suitable to improve the drought tolerance of wheat (Molnár et al. 2004). However, in the case of other *Aegilops* species there are hardly any data on the tolerance to abiotic stress factors like drought and heat stress (Rekika et al. 1977; Zahireva et al. 2001). In addition, diploide goat grasses, such as *Ae. tauschii* Coss. (DD), *Ae. bicornis* (S^bS^b) and *Ae. speltoides* Tausch. (SS) have some other advantages since the B and D genome of wheat are originated from *Ae. speltoides* and *Ae. tauschii* species (Jauhar and Chibbar 1999). Consequently, the chromosome mediated gene transfer from these species to hexaploide wheat is easier than from *Ae. biuncialis*. Moreover, several PCR based molecular markers specific to the B or D genome could facilitate the isolation of genes responsible for drought tolerance in these goat grasses (Röder et al. 1998). It follows that it is worth looking for accessions of various *Aegilops* species with good drought and heat tolerance that are more closely related to wheat than *Ae. biuncialis*.

Ae. speltoides and *Ae. tauschii* are wide-spread in the western Asiatic region and the latter in Central Asia, as well; *Ae. bicornis* grows in North Africa characterised by hot summers with a low amount of seasonal or annual rainfall. Although the *Aegilops* plants mentioned above are able to effectively survive these unfavorable environmental factors, no information has been provided on their drought and heat tolerance.

Drought and heat are important biomass-limiting stress factors (Berry and Björkman 1980; Araus et al. 2002) in the field causing the suppression of cultivated plants in growth and in crop production (Blum et al. 1997). During drought the water potential (ψ), relative water content (RWC) and net photosynthetic CO_2 fixation (A) substantially decrease (Bajji et al. 2001; Molnár et al. 2004). The reduction of A partially results from the closure of stomata due to water deficit, since decrease of stomatal conductance (g_s) is the most efficient way to reduce water loss, and parallel with this the CO_2 diffusion into the leaves is restricted, resulting in a decrease in intercellular CO_2 concentration (C_i) (Cornic 2000). On the other hand, the limitation of CO_2 fixation during water deficit is also influenced by the diffusion of CO_2 from the intercellular spaces to chloroplasts (Delfine et al. 1999; Loreto et al. 2003; Molnár et al. 2005), and by other metabolic factors such as changes in the capacity of ribulose-1,5-bisphosphate-carbosilase-oxigenase (Rubisco) and perturbed regeneration of ribulose-1,5-bisphosphate, etc (Medrano et al. 1997; Maroco et al. 2002; Centritto et al. 2003; Chaves et al. 2003; Molnár et al. 2004).

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The heat sensitivity of plants is closely connected to the thermal stability of PSII. It is more or less clear that the thermal tolerance of the photosynthetic apparatus in some higher plants is influenced by other stress factors like light (Havaux and Tardy 1996; Molnár et al. 1998), and by water deficit in a desiccation tolerant moss (Dulai et al. 2004). The study of these problems is further justified by the fact that under natural conditions high light intensity, heat stress, and water deficit occur in combination with each other: the effects of the three stress factors need to be tolerated at the same time.

In connection with the above-mentioned facts the examined *Aegilops* species are natives in the Mediterranean and in Asiatic arid or semi-arid continental regions, which are characterised by hot vegetation periods with a low amount of rainfall. On the other hand, physiological acclimation features in some measure depend on the climate of the original habitat of plants (Zahireva et al. 2001; Bultynck et al. 2003; Molnár et al. 2004). Since the vegetation period in native habitats of the examined *Aegilops* species is dry and hot, these plants had to develop various acclimation strategies to drought and to heat.

In this paper we compare some photosynthetic responses to drought and heat in three diploid *Aegilops* species originating from arid habitats with two wheat genotypes presumably characterised by a different drought tolerance to indicate that some of them have better drought tolerance with low temperature sensitivity than wheat, making them suitable for improving the drought and heat tolerance of wheat by intergeneric crossing, enabling it to survive the dry and hot periods in the field.

Materials and Methods

Plant materials

Aegilops species originating from areas with differing annual rainfalls (*Ae. speltoides* MvGB 1042 450-1450 mm, *Ae. tauschii* MvGB 605 100-350 mm and *Ae. bicornis* MvGB 585 75-275 mm) were provided by the gene bank of the Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, Hungary). The winter wheat (*Triticum aestivum* L.) Mv9kr1 has presumably moderate drought tolerance and Sakha is drought tolerant (Trivedi et al. 1991). The examined *Aegilops* lines were selected previously from thirteen accessions by germination ability in 15% (w/v) polyethyleneglycol solution. All experiments were performed on intact leaves or leaf segments of *Triticum aestivum* and of *Aegilops* sp. Seeds were germinated under laboratory conditions. After germination, these plants were grown in 1.5 kg soil pots in an unheated greenhouse for 5 weeks under natural sunlight. The water deficit was induced by withholding the water supply in the soil. The water status of plants was traced by determining the relative water content (RWC) according to the following equation: RWC = (FW – DW) X 100 / (SW – DW) where FW is the fresh weight, SW the water saturated weight and DW the dry weight after drying for 12 h at 105°C.

Chlorophyll a Fluorescence Measurements

The *in vivo* chlorophyll a fluorescence was measured in dark-adapted intact leaves with a pulse amplitude modulation fluorometer (PAM 101-103, Walz, Effeltrich, Germany) as described by Dulai et al. (1998), and recorded with a potentiometric chart recorder (NE-244, EMG, Budapest, Hungary) and a computer. The initial level (F_0) of fluorescence was excited by a weak 650-nm light beam modulated at 1.6 kHz (0.01 μmol m^{-2} s^{-1}). The fluorescence was detected by a PIN S1723 photodiode. The maximal fluorescence level (F_m) of the dark-adapted leaves was induced by a white saturating flash (4000 μmol m^{-2} s^{-1}) of 0.8 s duration, provided by a Schott KL-1500 light source (Schott, Essex, UK). Photosynthesis was induced for 15 min by continuous actinic light of 1000 μmol m^{-1} s^{-1}. The variables and equations for quenching analysis were determined according to van Kooten and Snel (1990). The quantum efficiency of photochemistry was calculated as $\Delta F/F_m'$, as described by Genty et al. (1989).

Heat-induced Chlorophyll Fluorescence

For the determination of the breakpoints (T_c,) of initial (F_0) and steady-state (F_s) fluorescence vs. temperature (T) curves the method of heat induction of fluorescence was applied as described by Schreiber and Berry (1977). The leaves were dark-adapted for 30 min, and then placed on the thermoelectric module. During heating from 25°C to 60°C at a rate of 1°C min^{-1}, the temperature was monitored by a thermocouple thermometer. Heating for F_s vs. T curves was started when the photosynthesis was steady. T_c was determined from the F_s vs. T curves.

Gas exchange measurements

CO_2 assimilation of intact leaves was measured with an infrared gas analyser (LCA-2, Analytical Development Co. Ltd, Hoddesdon, UK) in a semi-closed gas-exchange system. The white light for excitation of photosynthesis was provided by a Schott KL-1500 light source through a fiberopticable. The rates of net CO_2 fixation (A), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) fixation were calculated in the light saturated state of photosynthesis by using the equations of von Caemmerer and Farquhar (1981). The light response curve of A was determined in the range of 100-1200 μmol m^{-2} s^{-1}. The responses of A to changing in ambient CO_2 concentration was measured between 3-1000 ppm CO_2 at 1000 μmol m^{-2} s^{-1} light intensity using a gas diluter (Analytical Development Co. Ltd, Hoddesdon, UK). Stomatal (L_s) and mesophyll (L_m) limitation were determined on the basis of the A *vs.* C_i curves as described Lawror (2002).

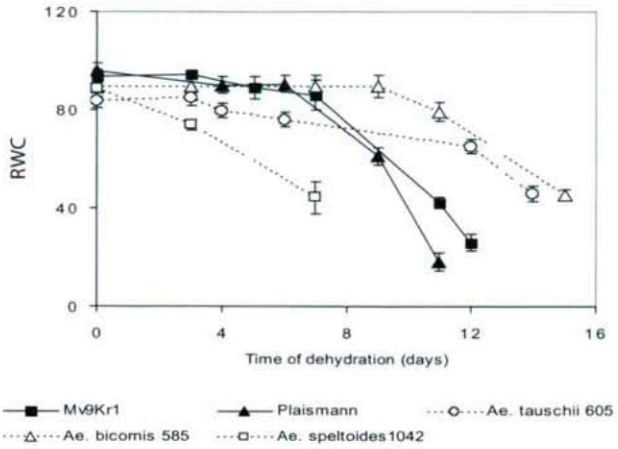

Figure 1. Effects of drought stress on relative water content (RWC) for wheat cultivars and for *Aegilops* species.

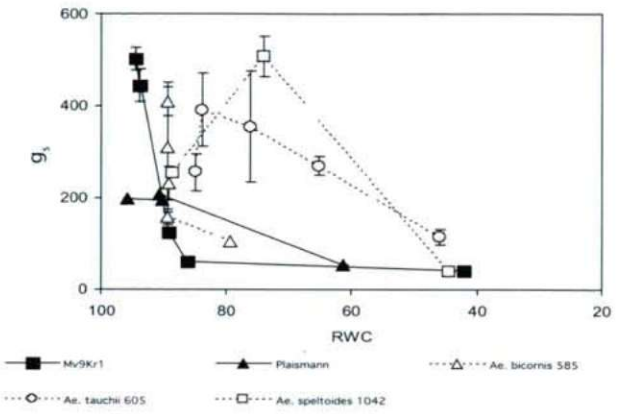

Figure 2. Effects of decrease in relative water content on stomatal conductance (g_s mmol H_2O m^{-2} s^{-1}) for wheat cultivars and for *Aegilops* species.

Statistical analysis

Student *t*-tests were performed using MS Excel (Microsoft Corporation, Seattle, USA). Differences between results are described as being significant where $P \leq 0.01$, and not significant where $P > 0.01$.

Results and Discussion

Effects of drought stress on the water content of the leaves

During drought stress, the water balance of plants is disrupted, as a result of which the RWC and water potential (ψ) of leaves decreases (Bajjii et al. 2001). In most cases stomatal closure can be observed, parallel with which stomatal conductance (g_s) decreases (Cornic 2000; Molnár et al. 2004) to reduce the water loss. If plants are able to hold the water effectively, that is, when the water potential is kept high in the dry period as well, they may have a good chance to survive the dry period, which however does not mean that the related physiological and photosynthetic processes are not susceptible to the low water content. After the withholding of water supply, the time dependence of RWC decrease was considerably different in *Ae. bicornis* MvGB 585 from that of wheat (Fig. 1). In this plant, water loss was substantially slower than in wheat cultivars, with a significant decrease of RWC only after the 9th-10th day, and its water content was significantly higher than that of wheat even at the end of the dry period. The originally high g_s decreased significantly at a slight water loss (Fig. 2), and stomatal closure, as is well-known, is the most efficient way of reducing transpirational water loss (Cornic 2000). It is interesting that *Ae. tauschii* MvGB 605, while efficiently keeping water, was not characterised by abrupt and significant stomatal closure; its RWC during drought did not decrease drastically, despite the higher g_s. As opposed to the ones mentioned above, it is a line in which water loss was faster than in wheat (*Ae. speltoides* MvGB 1042,). In this plant, under normal water conditions g_s was lower than in *Ae. bicornis* and wheat, but decreased less with water loss and can even increase at the beginning of the desiccation period. However, despite this fast and considerable decrease in RWC *Ae. speltoides* was capable of maintaining a relatively high rate of A until 70% RWC compared with wheat and *Ae. bicornis* MvGB585.

Effects of drought stress on the gas exchange and fluorescence induction parameters

It is well known that during water deficit stomata play an important role not only in the regulation of transpiration water loss but, also, a primary physiological effect of drought is the inhibition of photosynthetic CO_2 fixation partly by stomatal closure (Sharkey 1990; Chaves 1991; Cornic 1994; Molnár et al. 2004) g_s, though not to the same degree and not with the same RWC values, decreased in all examined genotypes with the decrease in RWC (Fig. 2). The strongest stomatal closure was detected in wheat cultivars, especially in Mv9kr1 and in *Ae. bicornis*. Contrary to this, stomata in *Ae. tauschii* and *Ae. speltoides* remained open in a wider range of RWC than those in wheat. When other factors do not limit the carboxylation processes, this decrease of stomatal conductance (g_s), which may restrict the diffusion of CO_2 into the leaves, has been reported to lead to a modification in intercellular CO_2 level and, as a result, to a decrease in photosynthetic CO_2 fixation (Flexas and Medrano 2002). As can be clearly seen in Figure 3 A was strongly restricted as RWC fell in the case of wheat cultivars and *Ae. bicornis*. However, despite the water loss *Ae. speltoides* and *Ae. tauschii* were capable of maintaining a satisfactory rate of net photosynthesis even at lower RWC values. These results indicate that the latter two goat grasses, similarly to some *Aegilops biuncialis* accessions (Molnár et

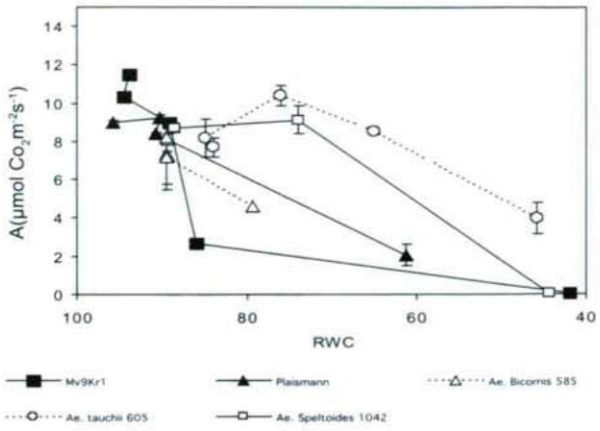

Figure 3. Effects of decrease in relative water content on net photosynthetic CO_2 fixation (A, μmol CO_2 m^{-2} s^{-1}) for wheat cultivars and for *Aegilops* species.

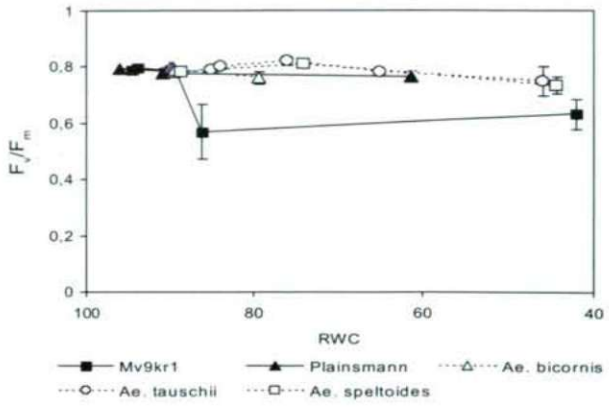

Figure 5. Effects of decrease in relative water content on optimal quantum yield (F_v/F_m) of PSII for wheat cultivars and for *Aegilops* species.

al. 2004), could retain their CO_2 fixation rate in spite of high water deficit.

In water-saturated C_3 plants, with environmental CO_2 concentration and corresponding C_i, at saturating light intensity, A does not reach the maximum level which is measurable at saturating CO_2 concentration (A_{max}). As suggested previously, stomatal closure is the main limitation to CO_2 fixation during drought since A_{max} can be recovered by a high CO_2 level (Cornic 2000; Cornic and Fresneau 2002); however, other studies have reported that the maximal rate of net photosynthesis is not fully recovered by the increased CO_2 concentration: decrease of A can also result from the reduced mesophyll conductance (Delfine et al. 1999; Flexas et al. 2002; Loreto et al. 2003) or important metabolic factors (Tezara et al. 1999; Delfine at al. 2001; Lawror and Cornic 2002; Centritto et el. 2003; Chaves et al. 2003). In accordance with the above mentioned facts the factors affecting photosynthetic CO_2 fixation during water deficit have been termed "stomatal" (L_s) and mesophyll (L_m) limitations (Lawror 2002). As can be seen in Figure 4 the extent of L_s decreased intensively as the RWC fell in wheat cultivars and in *Ae. bicornis*, while remained almost unchanged for *Ae. tauschii* and *Ae. speltoides* between 90 and 70% RWC. At lower RWC values (≤90%) L_m increased drastically in the case of wheat and *Ae. bicornis* indicating the primary role of the mesophyll limitation in the inhibition of A. Contrary to this, the relative importance

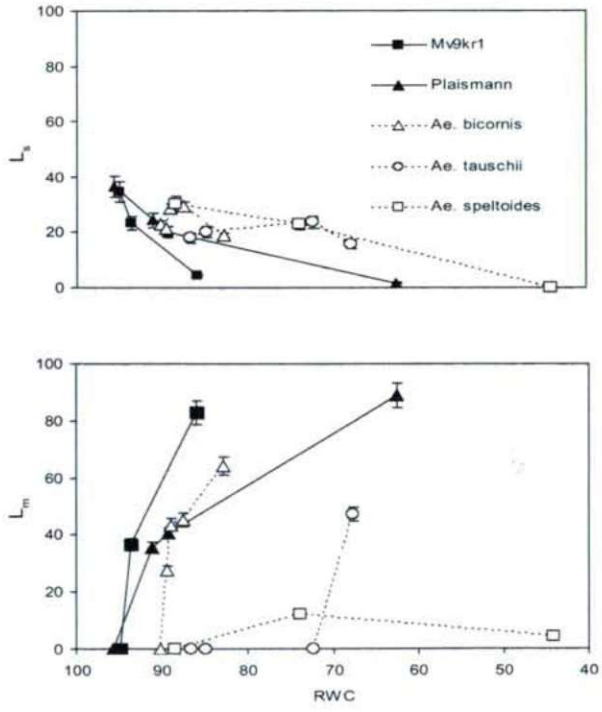

Figure 4. Stomatal (L_s) and mesophyll (L_m) limitations of net photosynthetic CO_2 fixation in relation to relative water content (RWC) for wheat cultivars and for *Aegilops* species.

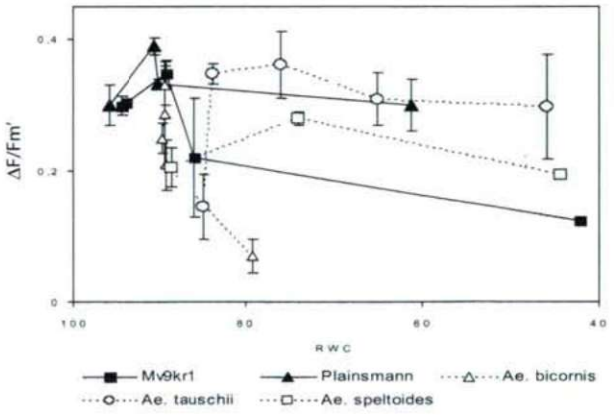

Figure 6. Effects of decrease in relative water content on effective quantum yield ($\Delta F/F_m'$) of PSII for wheat cultivars and for *Aegilops* species.

Table 1. Effect of water deficit on the breakpoints (T_c) of the F_0 vs. T (practically recorded in darkness) and F_s vs. T curves at 1000 μmol m^{-2} s^{-1} actinic light (AL) intensity. $T_{c\,dark}$, T_c values of F_0 vs. T curves in non-stressed plants; T_{c0}, T_c values of F_0 vs. T curves in non-stressed plants; T_{c1}, T_c values of F_0 vs. T curves measured at the end of the dry period.

Species, cultivars	$T_{c\,dark}$	T_{c0} (1000 μmol m^{-2} s^{-1})	T_{c1} (1000 μmol m^{-2} s^{-1})
Mv9Kr1	43,5±0,817	45,7± 0,173	49,0±0.000
Plaismann	42,7±0,251	46,2±0,251	47,5±0.500
Ae. tauschii 605	43,0±0,000	46,4±0,360	51,0±0.500
Ae. bicornis 585	43,4±0,655	46,8±0,404	48,0±0.000
Ae. speltoides 1042	43,0±0,642	46,5±0,286	49,0±0.000

of L_m was not significant in this range of RWC in *Ae. tauschii* and in *Ae. speltoides* (Fig. 4). These results indicate that L_m is the main limitation to photosynthesis in leaves of *Ae. bicornis* and wheat during water stress, attributed to the decrease of CO_2 diffusion from intercellular spaces to chloroplasts and the inhibition of some key metabolic processes. In spite of this, the processes in the background of mesophyll limitation are not significant in the case of *Ae. speltoides* and *Ae. tauschii* accessions. In the same way, a weak metabolic limitation has previously been reported based on oxygen sensitivity of photosynthesis in *Ae. biuncialis* accessions originating from arid habitats by Molnár et al. (2004).

Although, data have been published on the role of photochemical and electron transport processes (Keck and Boyer 1974; Giardi et al. 1996) in metabolic limitation, the optimal quantum yield (F_v/F_m) was not significantly affected by water deficit in the range 95-40% RWC (Fig. 5), as was also reported in other studies (Ben et al. 1987; Grieu et al. 1995). Therefore, our results suggest that drought has a marginal effect on the primary charge separation in the *Aegilops* species and in wheat cv. Plainsmann, whereas a slight decrease was observed in wheat cv. Mv9kr1 as the RWC fell. Nevertheless, already at medium water deficit the photosynthetic electron transport processes were significantly down regulated in Mv9kr1 as reflected in the decrease of effective quantum yield of PS II photochemistry ($\Delta F/F_m'$, Fig. 6). Contrary to this, $\Delta F/F_m'$ increased significantly in the case of *Ae. tauschii* and *Ae. speltoides* parallel with medium water deficit, probably indicating the increased proportion of open PS II centres, and it changed independently from the efficiency of primary charge separation (F_v/F_m). It is interesting that mesophyll limitation was also slight at this level of water stress in these plants while it became significant in wheat Mv9Kr1 and in *Ae. bicornis* parallel with the decrease of ($\Delta F/F_m'$).

On the basis of similar responses of g_s, A, L_s, L_m and photochemical efficiency it seems that besides some *Ae. biuncialis* accessions certain genotypes of *Ae. tauschii* and *Ae. speltoides* are also suitable to improve drought tolerance of wheat.

Heat tolerance changes of PSII during drought stress

The sensitivity of the photosynthetic apparatus to heat stress is closely connected to the thermal stability of PSII, which is well characterised by the critical values of the temperature dependence of the initial fluorescence level (F_0) of dark-adapted leaves (Schreiber and Berry 1977; Smillie and Nott 1979; Bilger et al. 1984). This method is also used to estimate heat tolerance of wheat and *Aegilops* species (Rekika et al. 1997). The heat tolerance of PSII in wheat and in *Aegilops* genotypes determined on the basis of the F_0 vs. T curves (practically in darkness) was not sufficient (42-43°C) for tolerating such high temperatures that are peculiar to their original habitats above the surface (Table 1) coupled with high irradiation and drought. Similarly to F_0, the breakpoints (T_c) of temperature dependence of steady state fluorescence (F_s) appropriately show the thermal stability of samples with a steady-state photosynthesis level (Molnár et al. 1998; Dulai et al. 2004). In connection with this, T_c values of F_s vs. T curves measured at moderately high AL intensity (1000 μmol m^{-2} s^{-1}) are shifted towards significantly higher temperatures (~46°C), indicating the higher thermal tolerance of PSII for wheat cultivars and for goat grasses (Table 1).

Although during drought the relative water content and the activity of some photosynthetic processes decrease, there are observations to the effect that in higher plants the slow dehydration of removed leaves resulted in an increase of the thermal stability of PSII (Havaux 1992). As a result of severe water deficit, in wheat cultivars and in examined goat grasses with steady-state photosynthesis at 1000 μmol m^{-2} s^{-1} AL intensity, the critical values of the F_s vs. T curves were shifted significantly higher, compared to the unstressed plants (Table 1). This enhanced thermal stability was more or less also manifested by the temperature dependence of the effective quantum yield of PSII (not shown by data). Although considerable differences were not detected between wheat and *Aegilops* accessions in these phenotypic responses (Table 1) to heat, these may be important in tolerating the high temperatures which occur during drought in their natural habitats.

On the basis of the results presented it seems that, although parallel with different degrees of water loss, *Ae. tauschii* MvGB 605, *Ae. speltoides* MvGB 1042 originated from dry habitats are able to maintain a sufficient CO_2 fixation and, at the same time, a high heat tolerance of the photosynthetic apparatus during drought. Besides, the slight mesophyll limitation of net photosynthesis in these plants may provide a good chance for the rapid recovery from a not too severe water stress. These properties make them a good candidate for improving the drought tolerance with low heat sensitivity of wheat by intergeneric crossing, to effectively survive the forecasted dry and hot periods in the fields of central Europe.

Acknowledgements

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ARTICLE

Effect of Chromium(VI) on growth, element and photosynthetic pigment composition of *Chlorella pyrenoidosa*

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ABSTRACT The effects of Cr(VI) were investigated on the growth rate, element, photosynthetic pigment and amino acid composition of *Chlorella pyrenoidosa*. Cr(VI) is toxic to Chlorella pyrenoidosa. The influence of chromium on cell density and cell number followed very similar trends, indicating that these growth responses might be correlated. The EC_{50} value for Cr(VI) were 2.0 mg dm^{-3}, the lethal concentration of chromium appears to be approximately 20 mg dm^{-3} for *Chlorella pyrenoidosa*. We have investigated the concentrations of chromium, calcium, magnesium and iron. The cells were fractionated into three fractions: cell wall fraction, membrane fraction, soluble fraction after the cells were disrupted. The amount of metals in whole cells and in each cell fraction was determined. Chromium uptake at each concentration was high within 3 days. *Chlorella pyrenoidosa* can accumulate chromium mainly (approximately 70%) in the cell wall. The concentrations of chromium and calcium show parallel changes with each other. A higher calcium concentration can be observed along with an increasing chromium concentration, both in the cell wall system and in the whole cells. Iron and magnesium concentration show a decreasing tendency. Cr(VI) caused a changes both in free amino acids and proline content. Both free amino acids and proline content increase with the increasing concentration of chromium. Chlorophyll a and b content show a decreasing , while OH-chlorophylls show increasing tendency. Rate of carotene β and α change grows with the increasing chromium concentration. The toxic properties of Cr(VI) can arise from the possibly free diffusion across cell membranes and strong oxidative potential. The toxicological impact of Cr(VI) originates from the action of this form itself as an oxidizing agent, as well as from the formation of free radicals during the reduction of Cr(VI) to Cr(III) occuring inside the cell. **Acta Biol Szeged 50(1-2):19-23 (2006)**

KEY WORDS

Chlorella pyrenoidosa
chromium
element composition
pigment pattern

Chlorella pyrenoidosa is an unicellular green alga, which is found in both fresh and marine waters. Its physiology, biochemistry and photosynthetic apparatus are similar to higher plants but its growth is very quick. For these reasons *Chlorella* is often studied in various metabolic and stress investigations (Rachlin and Grosso 1993; Lustigman et al. 1995).

When the concentration of a metal ions in the environment rises above a specific threshold heavy metal ions inhibit a variety of metabolic activities and prove toxic to most organisms. Interest in chromium originates from widespread use of this metal in various industries, such as metallurgical (steel, ferro and nonferrous alloys) and chemical (pigments, electroplating, tanning, others). Due to industrial run-off, process, large quantities of Cr compounds are discharged in liquid, solid and gaseous wastes into the environment, resulting in significant adverse biological and ecological effects (Kabata-Pendias and Pendias 2001). Chromium can exist in several chemical forms, displaying oxidation numbers from 0 to VI. Only trivalent and hexavalent chromium, are stable enough to occur in the environment. Cr(IV) and (V) form unstable intermediates in reactions of trivalent and hexavalent oxidation states with oxidizing and reducing agents respectively (Ball and Nordstrom 1998; Shriver et al. 2001). Cr(III) is the best known form displaying stability at neutral pH, if the complexation can be neglected. Under redox and pH conditions normally found in natural systems, chromium is removed from the solution as $Cr(OH)_3$, or in the presence of Fe(III) in the form of $(Cr_xFe_{1-x})(OH)_3$ where the x is the mole fraction of chromium (Sass and Rai 1987). Cr(III) generally has a lower toxicity than Cr(VI) compounds.

The Cr(III) is known to be essential for men and other mammals through its important function in glucose and lipid metabolism (Mertz 1975; Anderson 1989). Cr(VI) forms several oxygen associated species, the relative proportions of which depend on both pH and total chromium (VI) concentration. Within the normal pH range in natural waters CrO_4^{2-},

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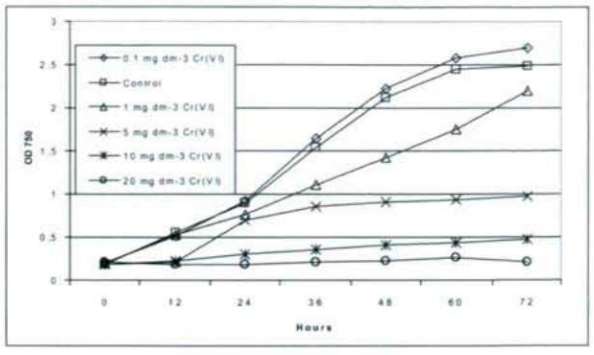

Figure 1. Effects of Cr(VI) on cell density of *Chlorella pyrenoidosa* in synthetic medium.

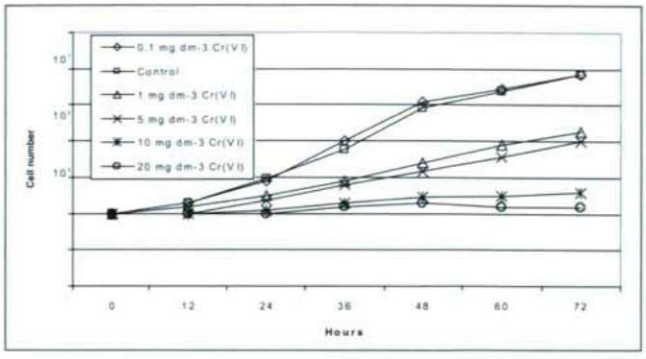

Figure 2. Effects of Cr(VI) on cell number of *Chlorella pyrenoidosa* in synthetic medium.

$HCrO_4^-$ and $Cr_2O_7^{2-}$ ions are the forms generally found and constitute many of the chromium (VI) compounds. They are quite soluble and mobile in the environment (Nieboer and Jusys 1988). Several laboratory studies have dealt with toxic effects of chromium in higher plants and algae (Wong and Chang 1991; Bishnoi et al. 1993).

The effect of Cr(VI) on the growth of *Chlorella pyrenoidosa* at different concentrations was studied under laboratory conditions. We also studied the element and photosynthetic pigment composition of algae, intracellular distribution of chromium and the levels of free amino acids and proline.

Materials and Methods

The algae used in this study were *Chlorella pyrenoidosa* (strain *IAM-C128*) obtained from the collection of the Institute of Applied Microbiology, University of Tokyo (Japan). Chemicals were purchased from Sigma Chem. Co. (St. Louis, USA), and Serva Fine Biochem. Gmbh. (Heidelberg, Germany).

Algae were maintained on agar. Cells were grown in sterile tubes containing synthetic media modified C-30. Cultures were aerated by filtered air bubbled with 5% CO_2, which served as a carbon source and stabilized the algal suspension homogeneity and pH at 7.2. Cells were permanently illuminated with white fluorescent light (18 Wm^{-2}) and were kept at 25°C during the growing period. The cultures were grown for 3 in some case 4 days. When cultures reached approximately $1x10^5$ cells/ml in the nutrient medium, algae were treated with 0.1 - 50 mg dm^{-3} of chromium (VI). We used $K_2Cr_2O_7$ as hexavalent chromium. For each experiment a control was also prepared of untreated *Chlorella pyrenoidosa* cells kept at the same conditions. Algae were autotrophically propagated for 72 hours after chromium treatments. The growth rate of algae cultures was followed by indirect turbidometric assay, and direct count using hemocytometer. Cells were collected by centrifuging (5000g x 10 min), and were washed 2 times with deionized water.

For algal cell fractionation cells were harvested by centrifugation at 5000g x 10 min, were washed twice with deionized water and centrifuged, and the weight of the cells was determined. This type of sample was considered as whole cells (sample 1). These prepared cells were disrupted using French press at 1500 kgf/cm^2 three times. French press is the most useful tool for disrupting *Chlorella* (Takeda and Hirokawa 1984). The homogenate was centrifuged at 3000g x 20 min and the pellet was allowed to stand in 1 ml of 0,5% sodium n-dodecyl sulphate (SDS) for 30 min. The homogenate with SDS was centrifuged at 10000g x 20 min to remove the soluble component, and the pellet was boiled in 80% ethanol for 20 min. The cell wall fraction was prepared by centrifuging the ethanol boiled pellet at 10000g x 20 min (sample 2). The miscellaneous fraction was prepared as the mixed supernatant after the treatment with SDS and ethanol (sample 3). The homogenate was centrifuged 15000g x 45 min after disruption of cells. The pellet was the membrane fraction (sample 4) and the supernatant was the soluble (cytoplasmic; sample 5; Okamura and Aoyama 1994).

The water of the supernatants was evaporated before digestion. Samples were digested for a day in concentrated HNO_3 and 30% H_2O_2 mixture (6:1 v/v rate), and dried. The mineralized residue was redissolved in 5 ml of 2N HNO_3 solution. The element composition was measured by a Spectroflame-type inductively coupled plasma atomic emission spectrophotometer (ICP-AES; Spectro Gmbh Kleve, Germany) with the following parameters: plasma gas 1.6 dm^3min^{-1}, nebulizer gas 0.6 dm^3min^{-1}, coolant gas 15 dm^3min^{-1}, excitation 27 MHz, 1.05 kW cross flow nebulizer.

When individual photosynthetic pigments were separated by HPLC technique algae pigments were extracted with a mixture of chloroform – acetone – isopropyl alcohol (2:1:1 v/v) at 4°C, the analytical procedure was detailed formerly (Simon et al. 1989). To determine free amino acid and proline content 200 mg fresh algae were shaken in 2 ml of 7% trichloroacetic acid for two hours, then they were filtered through paper filter and membrane filter (0.45 μm). The analysis was carried out using Biotronik LC 3000 amino acid analyzer

Table 1. Element composition of algal cell and cell fractions grown without and in the presence of 1, 5 or 10 mg dm^{-3} of Cr(VI) (the values are given in mg dm^{-3}).

	Control				1 mg dm^{-3} Cr(VI)			
	Cr	Ca	Mg	Fe	Cr	Ca	Mg	Fe
Whole alga cell	0,2	38±1	1423±54	148±4	17±1.8	64±5	1116±82	102±4.8
Cell wall fraction	0,2	45±1.4	1512±105	114±7.5	14±2.6	60±7.5	972±58	114±7
Membrane fraction	Nd	4.8±0.4	111±14	32±2.6	2.5±0.8	1.4±0.6	98±21	26±3.6
Soluble fraction	Nd	3.4±0.4	102±7	41±4.9	2.1±0.7	1.7±0.4	68±19	10.5±2.8
	5 mg dm^{-3} Cr(VI)				10 mg dm^{-3} Cr(VI)			
	Cr	Ca	Mg	Fe	Cr	Ca	Mg	Fe
Whole alga cell	28±3.6	74±4.2	981±104	78±8	24±3.8	79.5±4.2	981±81	62±5.1
Cell wall fraction	28.8±1.5	77±4.9	932±71	82±3.8	31±4.2	81.2±3.7	712±88	79±4.2
Membrane fraction	4.1±0.9	6.4±1.1	144±22	11±2.1	2.1±0.9	7.5±1.1	94±7.6	10.2±1.4
Soluble fraction	3.4±1.2	2.8±1	49±11	2.5±0.9	2.8±0.4	3.2±1.2	51±4.9	3.2±1.3

Nd: not detectable.
Values represent the mean ± SD of one experiment in triplicate.

(Galiba et al. 1992). The results are the means of 3-5 replications for each treatment. Three independent repetitions were performed for each experiment. The data were statistically evaluated calculating the standard deviation, and by statistical analysis using Tukey's *b*-test.

Results

The effects of chromium (VI) on *Chlorella pyrenoidosa* was investigated using increasing concentration of chromium from 0 to 50 mg dm^{-3}. As seen in Figures 1 and 2, except for a slight increase in growth of the algal cells at 0.1 mg dm^{-3} in comparison to the control, an increase in chromium concentration caused a significant decrease in the cell density and cell number. The influence of chromium on cell density and cell number followed very similar trends, indicating that these growth responses might be correlated. The EC_{50} value for chromium (VI) based on inhibition of cell growth was 2.0 mg dm^{-3}. The cell cultures treated with Cr(VI) from 20-50 mg dm^{-3} showed chlorotic symptoms, and the cells did not grown when we transferred them to fresh medium without chromium. This suggests, that the lethal concentration of Cr(VI) appears to be approximately 20 mg dm^{-3} for *Chlorella pyrenoidosa.*

The concentrations of 21 elements (Al, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, S, Sr, Ti, V, Zn) were determined in algae cells, and the concentrations of chromium, calcium, magnesium, iron are presented in Table 1. The cells were fractionated after disruption to cell wall fraction, membrane fraction, and soluble fraction. The concentrations of metals in whole cells and in each cell fractions were determined. Within 3 days chromium uptake at each concentration was high indicating that algal cells are able to bind and accumulate chromium. Generally 70% of the chromium is localized in the cell wall region, while the amount of accumulated chromium was almost the same in the membrane and the soluble fraction.

Comparison the data of the control and treated cells demonstrates the high rate of accumulation of chromium in the cells (Table 1). The chromium content of control cells is 0.2 mg dm^{-3}, while the values of treated cells are a hundred times higher. Intracellular distribution of chromium is heterogenous in different fractions. The concentrations of chromium and calcium show similar changes. An increasing chromium concentration causes higher calcium concentration, both in the cell wall system and in the whole cells. The increased Ca concentration is surprising, because an ion exchange would be expected. Both iron and magnesium concentrations show de creased concentrations with increased chromium concentrations.

All concentrations of chromium produced changes in both free amino acids and in proline concentrations as seen Figure 3 and 4. Increasing the concentration of chromium to 10 mg dm^{-3} increased the concentration of free amino acids and proline. At higher concentration of chromium the amount of amino acid concentration decreased. Proline concentration is known to increase in different type of stresses such as salinity, drought, low and high temperature, and heavy metals (Ibarra-Caballero et al. 1988; Kavi Kishor et al. 1995). The toxic properties of Cr(VI) may arise from free diffusion across cell membranes and strong oxidative potential.

The toxicological impact of Cr(VI) originates from its action as an oxidizing agent, as well as from the formation of free radicals during the reduction of Cr(VI) to Cr(III) occuring inside the cell (Nieboer and Jusys 1988). Decreasing levels of free amino acids and proline at higher concentration of chromium maybe due to the degradation of the cells.

Table 2. Photosynthetic pigments' concentration of algal cells growth with and without Cr(VI).

	Control	Cr(VI) 1 mg dm^{-3}	5 mg dm^{-3}	10 mg dm^{-3}	20 mg dm^{-3}
chlorophylls					
chlorophyllid b	0,028	0,024	0,024	0,020	0,018
chlorophyllid a	0,350	0,250	0,256	0,284	0,126
chlorophyllid a'	0,122	0,086	0,074	0,078	0,082
OH-chlorophyll b-1	0,052	0,070	0,164	0,232	0,258
OH-chlorophyll b-2	0,046	0,082	0,144	0,208	0,220
chlorophyll b	0,842	0,872	0,878	1,352	0,734
chlorophyll b'	0,246	0,272	0,220	0,248	0,182
OH-chlorophyll a-1	0,116	0,158	0,238	0,268	0,276
OH-chlorophyll a-2	0,084	0,202	0,308	0,316	0,304
chlorophyll a	4,338	4,574	4,052	3,808	3,404
chlorophyll a'	0,616	0,756	0,510	0,522	0,580
pheophytin a	0,036	0,090	0,138	0,170	0,228
carotenoids					
violaxanthin	0,160	0,192	0,176	0,202	0,138
anteroxanthin	0,490	0,540	0,458	0,584	0,420
lutein	1,324	1,372	1,390	1,830	1,960
xantophylls*	0,202	0,248	0,344	0,402	0,304
α-criptoxanthin	0,098	0,136	0,130	0,088	0,036
β-criptoxanthin	0,048	0,048	0,034	0,018	0,016
α-carotene	0,198	0,154	0,158	0,150	0,176
β-carotene	0,344	0,274	0,348	0,368	0,462

xantophylls* : unidentified xantophylls

The effects of chromium on concentration of photosynthetic pigments and chlorophyll derivatives are seen in the Table 2. The amounts of both chlorophyll-a and chlorophyll-b decrease with increasing chromium concentration, although the decrease in chlorophyll-a is larger than in chlorophyll-b. Conversely, the amounts of both OH-chlorophyll-a and OH-chlorophyll b (oxidative products of the chlorophylls) increased with the increasing concentration of chromium.

When Chlorella green algae were grown under similar conditions to this experiment, addition of metal ions (namely titanium, gallium and zirconium) to growth medium also caused changes in photosynthetic pigment concentration and composition. Appearance of new chlorophyll derivatives was observed in titanium, gallium and zirconium treated Chlorella cultures (Simon et al. 1988; Simon et al. 1989; Simon et al. 2001). Decomposition of chlorophylls and forming of chlorophyll derivatives could be attributed to in vivo action of enzymes like peroxidase (Kato and Shimizu 1985; Simon et al. 1989). Since peroxidase is located mainly in the vacuoles, and chlorophyll is compartmentized in the chloroplast, presumably peroxidase decomposes chlorophyll in vivo in the processes where membranes are dis-integrated (Kato and Shimizu 1985). We suppose that higher concentration of Cr(VI) acts indirectly to chlorophyll metabolism (*i.e.* by stimulation the dis-integration of membranes, and enhancing the activity of peroxidase), and causes appearance of chlorophyll derivatives as OH-chlorophyll-a and OH-chlorophyll-b.

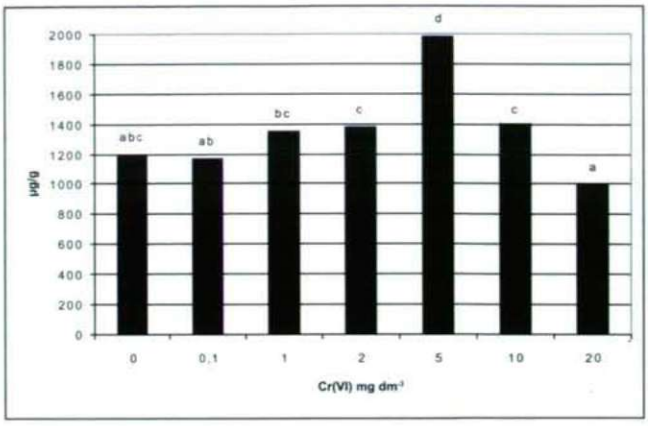

Figure 3. Total amino acid content in *Chlorella pyrenoidosa* grown in synthetic medium treated with Cr(VI). Statistical analysis was done by Tukey's b-test. Data are means of 3 replications. Bars of means signed by the same letter are not statistically significant at P=0.05.

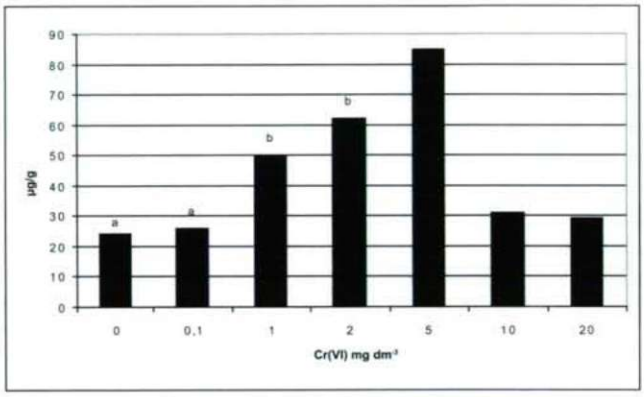

Figure 4. Proline content in *Chlorella pyrenoidosa* grown in synthetic medium treated with Cr(VI). Statistical analysis was done by Tukey's b-test. Data are means of 3 replications. Bars of means signed by the same letter are not statistically significant at P=0.05.

There were also changes in carotenoid levels. In the control cells the ratio of β - α carotene is about 1.5, but the rate became higher with the increasing chromium concentration. This phenomenon could be a protecting process against heavy metal induced oxidative stress (Salguero et al. 2003).

Discussion

Cr(VI) is toxic to *Chlorella pyrenoidosa*. *Chlorella* green algae can accumulate chromium mainly in the cell wall. Both free amino acids and proline content increased with the increasing concentration of chromium in the growth medium. Chlorophyll a and b decreased, while OH-chlorophylls increased in cells. Ratio of carotene β - α increased with the

increasing chromium concentration. These data suggest that the toxic effects of chromium on green algae originates from the action of Cr(VI) as an oxidizing agent as well as from the formation of free radicals during the reduction of Cr(VI) to Cr(III) occurring inside the cell. The toxic properties of Cr(VI) may arise from the free diffusion across the cell membranes and strong oxidative potential.

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ARTICLE

Pyrophosphate:fructose 6-phosphate 1-phosphotransferase operates in net gluconeogenic direction in taproots of cold and drought stressed carrot plants

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ABSTRACT The purpose of this work was to further investigate the regulatory interplay between pyrophosphate:fructose 6-phosphate 1-phosphotransferase (PFP) and its positive effector fuctose 2,6-bisphosphate (Fru-2,6-P_2) in heterotrophic tissues. Transformation of carrot plants (*Daucus carota* L. cv. Nantes Duke) with mutated mammalian 6-phosphofructo-2-kinase / fructose 2,6-bisphosphatase gene (6-PF-2-K/ Fru 2,6-P_2ase) produced carrot taproots which possessed between 163% and 410% of the Fru-2,6-P_2 levels observed in wild-type taproots. Besides Fru-2,6-P_2, the levels of 3-phosphoglycerate (3PGA) and hexose phosphates (hexose-P) showed the most significant alterations. Transgenic taproots possessed a marked increase in PFP activity that was accompanied by high 3PGA / hexose-P ratios under normal physiological conditions. Interestingly, 3PGA / hexose-P ratios became significantly lower in taproots exposed to drough or cold without any decrease in PFP activity. We suggested that changes in 3PGA / hexose-P ratios are a direct result of stimulation of PFP activity by the elevated Fru-2,6-P_2 levels. The Fru-2,6-P_2-stimulated PFP operates in the glyconeogenic direction in the taproots of stressed carrot plants, whereas the glycolytic direction dominates in the non-stressed controls. This suggests that the metabolic status determining the net activity of PFP depends on the physiological stress situations and such, PFP is an important sensor of the environmental changes. Likely that PFP is also involved in mobilisation of energy reserves upon unfavourable environmental changes by promoting the re-synthesis of transportable sucrose through gluconeogenesis from accumulated starch in taproots. **Acta Biol Szeged 50(1-2):25-30 (2006)**

KEY WORDS

PFP
fructose 2,6-bisphosphate
abiotic stress
glycolysis
gluconeogenesis
Daucus carota
taproot

Fru-2,6-P_2 is an important regulator of photosynthetic carbon metabolism (for review see Stitt 1990 and Nielsen et al. 2004). In leaves this signal metabolite contributes both to the coordination of sucrose synthesis with the rate of carbon dioxide fixation, and to the control of partitioning of photosynthate between sucrose and starch (Scott et al. 1995; Truesdale et al. 1999; Toldi et al. 2002). The allosteric inhibition of cytosolic fructose-1,6-bisphosphatase (FBPase) by Fru-2,6-P_2 is central to the proposed mechanism by which this effector influences both of these processes (Stitt 1997).

In contrast, the role of Fru-2,6-P_2 in non-photosynthetic plant tissues is poorly understood (Fernie et al. 2001). By analogy with animal and fungal systems it is frequently suggested that Fru-2,6-P_2 may contribute to the regulation of glycolytic flux (Stitt 1990). The basis of the this proposal is that non-gluconeogenic plant tissues often lack a detectable cytosolic FBPase activity (Entwistle and ap Rees 1990). Therefore, any influence of Fru-2,6-P_2 on metabolism must be attributed to the modulation of the glycolytic pathway and such the respiration through the activity of PFP in non-photosynthetic tissues of plants. The reaction catalysed by PFP in the cytosol of plant cells is close to equilibrium *in vivo* under normal physiological conditions (Weiner et al. 1987) meaning that PFP contributes equally both to the gluconeogenic and glycolytic flux. However, when plants are subjected to environmental stressors this equilibrium can be modulated.

It is known that Fru-2,6-P_2 provides adaptive abilities by metabolic fine tuning that are advantageous under suboptimal growth conditions (Okar et al. 2001; Nielsen et al. 2004). The Fru-2,6-P_2 signalling system sensitively responds to salt, drought, cold and osmotic stress by adjusting the fuel homeostasis according to the changing demand for survival (Reddy 1996, 2000, Banzai et al. 2003, Villadsen et al. 2005). Storage organs like potato tubers and carrot taproots function as fuel reserves providing mobilisable energy sources under stress conditions. Fru-2,6-P_2 is involved in the regulation of diurnal turnover of starch, which is the most important mobilisable energy source in higher plants, and has pivotal role is stress adaptation. At the same time, PFP can substitute 6-phosphofructo-1-kinase (PFK) in maintaining glycolytic flux under ATP-limited stress situations by using PPi as phosphoryl

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donor. Since PFP is stimulated by Fru-2,6-P_2 allosterically, it is not an exaggregation to suppose that the functions of PFP and Fru-2,6-P_2 overlap in plant stress responses.

To validate this assumption, transgenic carrot plants with elevated levels of Fru-2,6-P_2 were produced and analysed. Taproots of these transgenic plants have been used to analyse whether (i) PFP activity is altered as a result of elevated Fru-2,6-P_2 levels and (ii) is there a change in the direction of the net activity of PFP when taproots are subjected to different abiotic stressors (drought, cold).

Materials and Methods

Plant material and growth conditions

In vitro grown carrot plantlets (*Daucus carota* L. cv. Nantes Duke) were obtained from dr. Seppo Sorvari (MTT Agrifood Research, Piikkio, Finland). After micropropagation and genetic transformation, transgenic and control plants were potted and grown in peat-soil in a growth chamber with supplemetary lighting of 12 h / 150 mmol photons m^{-2} s^{-1} at 24°C, and 70% relative humidity. Healthy, young taproots of 2 month old plants were used for physiological measurements. Stressed lines were cultured in two ways prior to analysis. While half of the potts were grown at 10°C for 10 days, the other half of the potts were grown without further irrigation also for 10 days. Plants were then re-watered and samples were taken for physilogical measurements at noon 1 day after re-watering.

Plasmid constructs

Functional 6-PF-2-K was provided by a modified coding region of the rat liver 6-phosphofructo-2-kinase / fructose 2,6-bisphosphatase gene (6-PF-2-K/ Fru 2,6-P_2ase). The gene contained point mutations which changed serine-32 and histidine-258 to alanine (Tauler at al. 1991; Kurland et al. 1992). These modifications result in a functional enzyme which possesses no Fru 2,6-P_2ase activity, but can still make Fru-2,6-P_2 (Scott et al. 1995). This construct was identical to that described by Scott et al. (1995). A *Hind* III fragment containing 6-PF-2-K/ Fru 2,6-P_2ase was inserted between a 35S CaMV promoter and polyadenilation signal in the vector pJIT62. The final construct was digested with *Kpn* I and *Eco* RV, then filled in, and cloned into pBIN19 (Bevan 1984). The pBIN19::6-PF-2-K construct was introduced into *Agrobacterium tumefaciens* strain LBA 4404, containing pAL4404, by direct transformation (Höfgen and Willmitzer 1988).

Plant transformation

Somatic embryos from cell suspension cultures of carrot plants (*Daucus carota* L. cv. Nantes Duke) were infected with the transforming *Agrobacterium*. Co-culture took place by immersing 2-3 mm long embryo segments into 40 ml *Agrobacterium* suspension that contained bacterial cells at OD_{660} = 0.5 density, one third-strength MS macro and microelements (Murashige and Skoog 1962), half-strength MS vitamins, 1.0 g l^{-1} casein hydrolysate, 100 µM acetosyringone and 10 g l^{-1} glucose (pH 5.0) for 20-30 minutes at 22°C under dim light. After infection, embryo segments were shortly dried and then placed to growth regulator-free MS medium solidified with 7 g l^{-1} plant agar for the following 2-3 days. After the 2-3 days co-culture, plant explants were transferred into selective callus induction medium that contained 1.0 mg l^{-1} 2,4-D, 100 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime. The selection took 6-8 weeks and required subculturing in every 2 weeks. Calluses were transferred into solid regeneration media that contained MS salts and vitamins, 1.0 mg l^{-1} zeatin, 50 mg l^{-1} kanamycin and 300 mg l^{-1} cefotaxime. The plant regeneration required 16 h daylength condition at 25°C. When *de novo* developed shoots reached 8-10 mm in length they were transferred onto solid MS media containing 1 mg l^{-1} IBA for rooting. Viable plants with well developed root system were potted in the greenhouse and were cultivated for further examinations.

Analysis of gene expression

In order to establish whether the transgene was present and expressed in putative transgenic carrot plants northern hybridisation was used. RNA was extracted from taproots using the methods described by Lichenstein and Draper (1985). Northern hybridisation was carried out as described by Sambrook et al. (1989).

Extraction and measurement on enzyme activity

For experimental material we concentrated on the non-photosynthetic taproots from the carrots. For measurements separate taproots were used for each experimental sample. Each of these taproots originated from separate clonal carrot plants.

Slices (ø = 10 mm x 2 mm thick) were cut from the centre of the taproot with a razor blade and immediately frozen in liquid nitrogen. A sample of about 500 mg FW^{-1} were then homogenised to a fine powder at 4°C in a mortar and pestle in the presence of 5 ml of extraction medium, 100 mM Hepes, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 5 mM β-mercaptoethanol (Hajirezaei and Stitt 1991). The homogenate was first centrifuged at 14000 rpm for 5 min, then the supernatant was assayed for 6-PF-2-K as described by Scott et al. (1995). These assay conditions were optimal for both the mammalian and the carrot enzyme. Other enzyme activities were measured as described in the following references: cytosolic FBPase (Hatzfeld et al. 1990; Hajirezaei and Stitt 1991) and ADPglucose pyrophosphorylase (Hajirezaei et al. 1994) PFP activites were measured first in glycolytic and gluconeogenic directions separately as described by Theodorou and Kruger (2001) and then total PFP activities were calculated by the addition of related data.

To check the reliability of the extraction and measurement

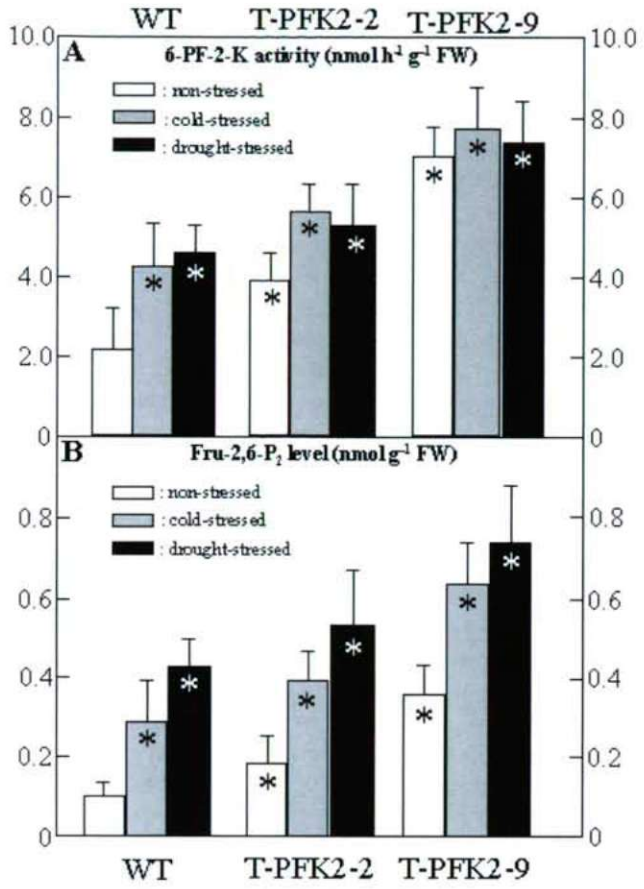

Figure 1. (i) Total activites of 6-phosphofructo-2-kinase (6-PF-2-K), responsible for synthetising Fru-2,6-P_2, and levels of Fru-2,6-P_2 in taproots of transgenic plant lines (T-PFK2-2 and T-PFK2-9) relative to WTs. (ii) The impact of cold and drought stress on the above data. Samples were harvested at noon from taproots of non-stressed control, as well as cold and drought stressed carrot plants. All data are the mean ± SD of replicate measurements of three separate taproots. Values labelled by asterisks are different significantly (Student's *t* test $P < 0.05$) from the corresponding data of non-stressed WT controls.

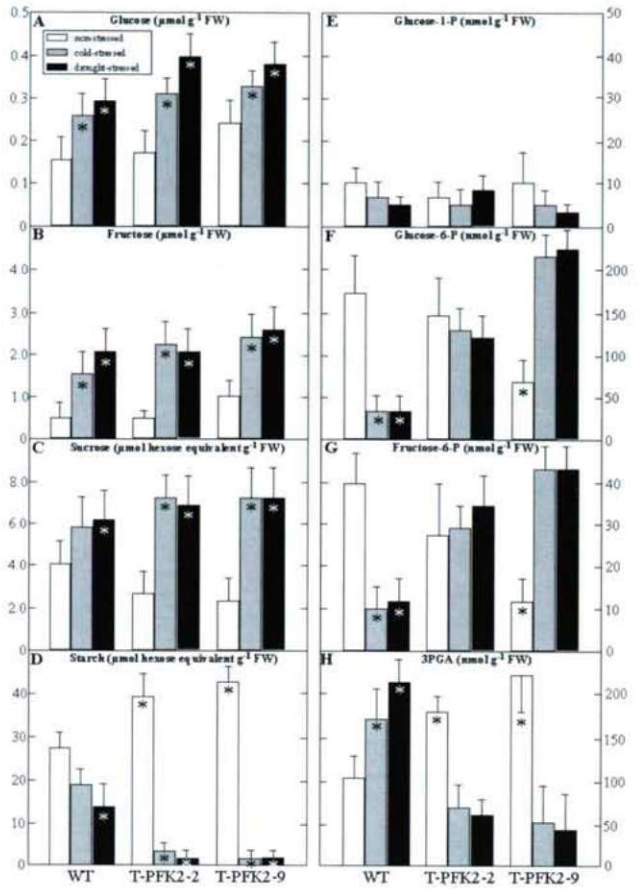

Figure 2. The content of soluble sugars, phosphorylated intermediates and starch in non-stressed (white bars), cold-stressed (grey bars) and drought-stressed carrot taproots with elevated levels of Fru-2,6-P_2 (lines T-PFK2-2 and T-PFK2-9) compared to WTs. Samples were harvested at noon from taproots. All data are the mean ± SD of replicate measurements of three separate taproots. Values labelled by asterisks are different significantly (Student's *t* test $P < 0.05$) from the corresponding data of non-stressed WT controls.

of 6-PF-2-K activity we performed recombination experiments. Spinach leaf extracts, containing approximately the same activity of 6-PF-2-K as was expected to be present in the taproots, were added to the samples prior to homogenisation.

Extraction and measurement of metabolites

Cores of tissue (10 mm x 10 mm, approximately) were removed from the centre of the taproots, and slices (2 mm thick) were cut and instantly frozen in liquid nitrogen. Samples (1 g FW^{-1}) were homogenised in liquid nitrogen in a mortar and pestle. For all substrates except Fru 2,6-P, the homogenate was immediately suspended in 2 ml of 1.4 M perchloric acid and left on ice for 2 h. Then the extract was neutralised with 5 M K_2CO_3, and the insoluble debris removed by centrifugation at 10000 g twice. Starch in the insoluble fraction was determined according to Morrell and ap Rees (1986). Sucrose and phosphorylated intermediates (3PGA, Glc-1-P, Glc-6-P, and Fru-6-P) were assayed in the soluble fraction by following enzyme-linked reduction of NAD^+ or oxidation of NADH spectrophotometrically at 340 nm according to Scott and Kruger (1995). Fru-2,6-P_2 was extracted from taproots and assayed as described by Scott and Kruger (1994). To confirm the reliability of the extraction and measurement of the metabolic intermediates we performed recovery experiments. This was done by adding to the sample, prior to extraction, an amount of the metabolite similar to that present in taproots. The recovery of metabolites added to the carrot tissue was 90.7 ± 5.0% for Suc; 88.7 ± 6.6% for Glc; 89.8 + 9.4% for Glc-6-P; 80.5 ± 4.5% for Glc-1-P; 69.2 + 8.7% for Fru-6-P;

89.1 + 3.6% for 3PGA and 83.2 ± 2.5% for Fru-2,6-P_2 (mean + SE, where n = 3).

Statistical analysis

Significance of differences between treatment groups was determined using Student's t-test followed by Fisher's LSD test as appropriate. If differences were considered significant for $P < 0.05$, means were separated by LSD at $P = 0.05$. Results for continuous variables are expressed as means ± SD.

Results and Discussion

In contrast to our clear understanding of the role of Fru-2,6-P_2 in the regulation of carbon partitioning during photosynthesis (Stitt 1990; Nielsen et al. 2004), the function of this signal metabolite in non-photosynthetic metabolism is equivocal (Fernie et al. 2001; Theodorou and Kruger 2001). Using transgenic tobacco plants containing Fru-2,6-P_2 levels which are up to double that measured in wild-type plants, evidence could be provided that this signal metabolite has an important role during starch mobilisation in tobacco levels in the dark (Scott and Kruger 1995). The rate of starch degradation in darkened tobacco leaves - used to model heterotrophic metabolism - is lower in the transgenic plants than in the wild-type plants. Through a combination of inhibition of the cytosolic FBPase and stimulation of PFP by increased Fru-2,6-P_2 levels, the amount of 3PGA in leaves is raised in the dark in the transgenic compared to the wild-type leaves (Scott and Kruger 1995). This rise correlates with an increase in the rate of unidirectional starch synthesis in the leaves. These data suggest that the net rate of starch degradation is reduced in the transgenic plants, because the elevated 3PGA levels stimulate starch synthesis through ADPglucose pyrophosphorylase. While such starch accumulation has not been observed in callus cultures of the above transgenic tobacco lines, the marked increase of 3PGA/hexose-P ratio in the same samples indicates a similar response to elevated Fru-2,6-P_2 levels in a different type of heterotrophic tissues (Fernie et al. 2001). On the contrary, no alterations in the 3PGA / hexose-P ratio were detected when the carbohydrate metabolism of potato tubers containing elevated levels of Fru-2,6-P_2 was compared to wild type controls (Rung et al. 2004).

As it can be seen from the above controversial conclusions, our understanding of the role of Fru-2,6-P_2 in heterotrophic plant tissues is still poor. More reliable observations can be made, if the regulatory role of Fru-2,6-P_2 in such tissues is evaluted considering the environmental aspects (Reddy 1996; 2000; Banzai et al. 2003; Villadsen et al. 2005). PFP is belived to be a typical sensor enzyme catalysing a near equlibrium reaction at the entering point of glycolysis (Stitt 1990). The metabolic consequence of being such sensor enzyme is that PFP activity is multimodulated by numerous positive and negative effectors. Environmental changes can be mirrored by dynamic changes in relative concentrations of these effectors and such, PFP becomes an indicator of optimal or suboptimal growth conditions.

The purpose of our work was dual. First, the regulatory interplay between PFP and its positive effector Fru-2,6-P_2 has been investigated in heterotrophic tissues. Second, we wished to check wether the integrated action of Fru-2,6-P_2 and PFP has any role in mobilizing energy from such storage organs like carrot taproots upon stress. Carrot somatic embryo explants were transformed with T-DNA containing the *npt* II and the 6-PF-2-K / Fru 2,6-P_2ase genes under the control of a CaMV 35S promoter. Kanamycin resistant plants which formed were then tested to confirm expression of the 6-PF-2-K / Fru 2,6-P_2ase gene. RNA was isolated and purified from leaves of putative transgenic lines. A radioactive probe for the 6-PF-2-K / Fru 2,6-P_2ase gene hybridized to the anticipated 1400 nucleotid transcript in all of the transformed plants, but did not hybridized to any message from wild-type plants (data not shown). In total, out of the twelve

Table 1. (i) Interrelation of the 3PGA/hexose-P ratio with the gluconeogenic and the glycolytic activity of PFP in taproots with elevated levels of Fru-2,6-P_2 (transgenic plant lines T-PFK2-2 and T-PFK2-9) relative to WTs. (ii) The impact of cold and drought stress on the above interrelation. 3PGA/hexose-P ratio was considered to be 1.0 in the case of non-stressed WT control. Samples were harvested at noon from taproots of non-stressed control, as well as cold and drought stressed carrot plants. All data are the mean ± SD of replicate measurements of three separate taproots.

Plant lines	Treatments	3PGA/hexose-P ratio	Enzyme activity [μmol min^{-1} g^{-1} FW] PFP gluconeogenic	PFP glycolytic	PFP total
WT	Non-stressed	1.00	0.09±0.02	0.11±0.02	0.20±0.04
	Drought-stressed	0.65	0.25±0.03	0.17±0.01	0.42±0.04
	Cold-stressed	0.70	0.26±0.04	0.14±0.02	0.40±0.06
T-PFK2-2	Non-stressed	1.25	0.17±0.01	0.21±0.02	0.38±0.03
	Drought-stressed	0.43	0.38±0.04	0.13±0.01	0.52±0.05
	Cold-stressed	0.26	0.41±0.02	0.10±0.01	0.51±0.03
T-PFK2-9	Non-stressed	1.29	0.19±0.03	0.24±0.02	0.43±0.05
	Drought-stressed	0.29	0.62±0.05	0.25±0.02	0.87±0.07
	Cold-stressed	0.31	0.67±0.04	0.15±0.02	0.82±0.06

kanamycin resistant plants tested, seven independent lines of transgenic plants possessed detectable levels of the appropriate transcript. As the result of successfull transformation, transgenic taproots possessed between 163% and 410% of the Fru-2,6-P_2 levels observed in wild-type taproots (Fig. 1). Besides Fru-2,6-P_2, the concentrations of 3-phosphoglycerate (3PGA) and hexose phosphates (hexose-P) showed the most significant alterations (Fig. 2). Transgenic taproots possessed a marked increase in PFP activity that was accompanied by high 3PGA / hexose-P ratios under normal physiological conditions (Table 1). Interestingly, 3PGA / hexose-P ratios became significantly lower in taproots exposed to drough or cold without any decrease in PFP activity. We suggested that changes in 3PGA / hexose-P ratios are a direct result of stimulation of PFP activity by the elevated Fru-2,6-P_2 levels and by cold and drought stress (Fig. 1, Table 1). The Fru-2,6-P_2-stimulated PFP operated in the glyconeogenic direction in the taproots of stressed carrot plants, whereas the glycolytic direction dominated in the non-stressed controls or in other heterotrophic tissues like darkened leaves (Scott and Kruger 1995) and tobacco calluses (Fernie et al. 2001). This suggests that the metabolic status determining the net activity of PFP depends on the physiological stress situations and such, PFP is an important sensor of the environmental changes. Likely that PFP is also involved in mobilisation of energy reservoires upon unfavourable environmental changes by promoting the re-synthesis of transportable sucrose through gluconeogenesis from accumulated starch in taproots (Fig. 2).

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Abbreviations

6-PF-2-K/ Fru 2,6-P_2ase = 6-phosphofructo-2-kinase / fructose 2,6-bisphosphatase gene; PFP = pyrophosphate:fructose 6-phosphate 1-phosphotransferase; PFK = 6-phosphofructo-2-kinase; Fru-2,6-P_2 = fuctose 2,6-bisphosphate; 3PGA - 3-phosphoglycerate; hexose-P = hexose phosphate; FBPase = cytosolic fructose-1,6-bisphosphatase; Suc = sucrose; Glc = glucose; Glc-1-P = glucose 1-phosphate; Glc-6-P = glucose 6-phosphate; Fru-6-P = fructose 6-phosphate; PPi = pyrophosphate

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ARTICLE

Isolated microspore culture of wheat (*Triticum aestivum* L.) with Hungarian cultivars

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ABSTRACT The most important steps of microspore-plant system were checked in isolated microspore culture of CY-45 spring genotype. A lot of microspore derived embryos were produced via embryogenesis and green and albino plantlets were regenerated from these structures. This is the first publication which reported about the androgenesis of numerous Hungarian agronomically important varieties in isolated microspore culture. Nine Hungarian varieties (GK Mini Manó, GK Garaboly, GK Hargita, GK Csongrád, GK Délibáb, GK Élet, GK Kata, GK Bán, Mv Palotás) were tested in isolated wheat microspore culture. Every genotype was responsive, embryos and embryo-like-structures were developed in cultures. Plantlets – albino and green -were regenerated in case of every Hungarian cultivar. Green plantlets were produced from six genotypes.

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KEY WORDS

cultivars
haploid
microspore culture
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Haploid plant production in anther culture of hexaploid (2n=6x=42) common wheat (*Triticum aestivum* L.) have been developed as a routine method in the last 25-30 years. *In vitro* wheat haploid induction in anther culture was published by Ouyang et al. (1973) in China more than 30 years ago. From the induced haploids to produce induced doubled haploids (DH) is a relatively easy and effective method using colchicine treatment (Jensen 1974). The genetically homozygous doubled haploids (DHs) in crop breeding would enhance its improvement by accelerating breeding programmes, improving selection efficiency. It means the research of haploid and doubled haploid production is in the highlight of breeders. In Europe, the DH lines of variety improvement at the end of the breeding process are very important tools in protection of intellectual property as a plant variety patent after DUS tests.

Nowadays, there are two plant biotechnology method supported ways to produce large number of haploids and DH plants: (i) anther culture and (ii) haploid production via wide (maize) cross (Szakács et al. 2002; Inagaki 2003). Today there is a new cell culture based approach under development including new advantages (direct study of embryogenesis from the first cell division, selection on cell level etc.). While the previously mentioned two methods are today routinely used in doubled haploid production systems, the isolated micropore culture technology basing upon direct embryogenesis of isolated microspores wheat is described as more recalcitrant.

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Laboratories are working with problem of microspore culture (Mejza et al. 1993; Tuvesson and Öhlund 1993; Zheng et al. 2001; Zheng et al. 2002), such as genotype dependency and albinism in regeneration of microspore derived pro-embryos of different cereal species (wheat, triticale, rice). But otherwise dicots as rapeseed (*Brassica napus* L.) and monocots as barley (*Hordeum vulgare* L.) can produce a lot of embryos in isolated micropore culture and thousends of green plants from regenerated embryos of microspore culture induced embryoids (Custers 2003).

Wheat is one of the most important small grain cereal of the world (Swaminatan 2001). This fact motivates biotechnologists to search new approaches - like haploid technology - in improvement methods. In literature, the first data on the isolated microspore culture-derived wheat plantlet can be found from Datta and Wenzel (1987). However, their regenerated plantlets were not real isolated microspore culture-derived ones, because they used float anther culture for induction of microspore embryogenesis. That phenomenon was an embryogenesis of shaded micropores in float anther culture. First reports of isolated microspore-derived wheat green plants were published by two different laboratories (Mejza et al. 1993; Tuvesson and Öhlund 1993) almost at the same time. Different methods – treatment with inducer chemicals, ovary-conditioned medium – were developed to improve the efficiency of embryo production of isolated microspores (Zheng et al. 2001; Zheng et al. 2002). Importance of ovary co-culture in successful microspore culture of wheat was first published by Mejza et al. 1993. They found a posi-

tive effect of ovary co-cultivation on embryogenesis in float anther culture of wheat. A significant positive effect of ovary co-culture was found by our laboratory, too (Puolimatka et al. 1996; Indrianto et al. 2001; Lantos et al. 2005).

In this paper we summarise the most important result of our laboratory in improving wheat microspore culture. The response of different wheat genotypes in isolated microspore have been in highlights.

Materials and Methods

Plant material

In our experiment, one spring wheat genotype (CY-45) and nine winter wheat genotypes were used to test androgenesis of Hungarian cultivars. Winter genotypes (GK Mini Mamó, GK Garaboly, GK Hargita, GK Csongrád, GK Délibáb, GK Élet, GK Kata, GK Bán, Mv Palotás) were grown in the nursery of Cereal Research Non-profit Company, Szeged, Hungary while spring type in greenhouse. The donor shoots were collected when the anthers of the middle-part of the spikes contained the microspores from mid- to late-uni-nucleate. All leaves were cut except the flag leaf. The tillers were put into Erlenmeyer flasks which contained fresh tap water and were covered by a PVC bag to assure high humidity. Donor tillers were cold pre-treated for about two weeks at 3-4°C. After cold treatment, the donor spikes were surface-sterilised for 20 minutes in 2% sodium hypochlorite solution using 2-3 drops 'Tween 20' solution. The spikes were rinsed three times with sterile water after sterilization.

Isolation of microspores from pre-treated anthers

Hudred and fifty anthers were isolated into 55 mm diameter plastic Petri dishes contained 5 ml 0.3 M mannitol solution and 200 mg l^{-1} antibiotic (cefotaxim SIGMA-ALDRICH C 7912, CAS No. 64485-93-4). The isolated anthers were cultured for three days at 32°C in the dark. Microspores were synchronized by osmotic pre-treatment and starvation.

After osmotic pre-treatment, wheat anthers consisted of a lot of microspores in late uni- and early bi-nucleate developmental stages. The anthers were homogenized by a glass rod upon a nylon filter (200 µm pore size) and the suspension was filtered again through 80 µm nylon sieve. The tissue and microspore suspension was centrifuged at 80 g for 5 min and resuspended in 2-3 ml 0.3 M mannitol solution. The viable microspores were collected by mannitol/maltose gradient centrifugation using 0.3 M mannitol and 21% maltose (SIGMA-ALDRICH M 5885 CAS No. 6363-53-7) solutions (Fig. 1a). The separated and cleaned microspores were washed in 0.3 M mannitol solution and centrifuged again at 60 g for 5 min. The collected microspores were re-suspended in culture medium. Viable microspores were counted using a Burker chamber. The density of isolated microspores was diluted to approximately 30-35 000 microspores ml^{-1}.

Culture of isolated microspores

Microspores were cultured into 35 diameter Corning plastic Petri dishes containing 1.5 ml culture medium. On the first three days, microspores were cultured at 32°C in the dark thermostat and given a hot stress treatment.

After heat shock, the cultures were kept in the darkness at 28°C, developing microspores and microspore-derived colonies were observed by CK-2 Olympus inverted microscope.

Wheat microspores of all genotype were cultured in modified W_{14} basic media (Jia et al. 1994) contained 1000mg/l Glutamin, 0.5 mg/l 2.4-D and 0.5 mg/l Kinetin- with ovary co-culture. Ten ovaries were put into every Petri dish.

Regeneration of green plants and their transfer to the greenhouse

Microspore culture derived structures were plated on the regeneration medium. Wheat pro-embryos were put on Gelrite (2.8 g l^{-1}) solidified 190-2Cu regeneration medium which contained 0.5 mg l^{-1} $CuSO_4 \times 5H_2O$ (Zhuang and Xu 1983; Pauk et al. 1991; Purnhauser and Gyulai 1993).

Green plantlets were transferred into glass tubes which contained the regeneration medium. Rooted plantlets were transferred to the greenhouse and wrapped with PVC bag during period of acclimatization.

Statistical analysis

Experiments were carried out in three replications at least and the data were analysed using appropriate programmes from the Minitab Release 14 statistical package (Minitab Inc.). In the table, the different alphabets after the dates mean significant difference at 95% probability.

Results

Isolated wheat microspore culture

The most critical steps of isolated wheat microspore culture were checked by cultivation of CY-45 genotype. The ideal microspore development stage is the first important step of androgenesis induction in microspore culture. The collected donor tillers consisted of microspores with mid-uni-nucleate stage in middle part of spikes.

After cold pre-treatment, the microspores of anthers were synchronised in 0.3 M mannit solution for 3 days at 32°C. Osmotic pre-treatment and starvation increased the number of viable isolated microspores. These stress pre-treatments changed the gametophytic pathway to sporophitic. After treatments, the microspores were in late uni- and early-bi-nucleate microspore stage (Fig. 1b).

The first cell divisions were obtained at the third and fourth days of culture in the isolated microspore culture. The sister cells inside the microspore wall could be observed

under an inverted microscope at the end of the first week of culture (Fig. 1c). Some structures died when their cells got away from microspore wall (Fig. 1d) but a lot of multi-cellular structures overgrew the microspore wall on the second week of culture (Fig. 1e). Most of them grew very intensively. The haploid microspore-plant system is based on the using of ovary co-cultivation which protected the developing structures against the death (Fig. 1f). After one month, microspore–derived embryos and embryo-like structures were developed in liquid medium (Fig. 1g).

The induced pro-embryos were plated on the solidified regeneration medium and green, albino plantlets were regenerated on the first or second week of regeneration (Fig. 1h). The green plantlets were well rooted and shooted in individual glass tubes (Fig. 1i). The plantlets were transplanted into greenhouse and grown to maturity (Fig. 1j). On the harvested plants, three different kind of spikes were found: fertile, partial fertile and sterile (Fig. 1k).

Isolated wheat microspore culture with Hungarian cultivars

The efficiency of this culture system was studied by nine Hungarian cultivars (GK Mini Manó, GK Garaboly, GK Hargita, GK Csongrád, GK Délibáb, GK Élet, GK Kata, GK Bán, Mv Palotás) and CY-45 spring wheat.

In case of every genotype, androgenesis was induced in isolated microspore culture with ovaries. The microspores developed very intensively and different numbers of embryoids were counted in the cultures of cultivars. Best results were achieved by CY-45, GK Élet, GK Csongrád and GK Mini Manó genotypes but the other varieties also produced numerous embryos (Table 1).

The developed embryoids were plated on the regeneration medium and the regeneration rate of cultivars was checked. Plantlets were regenerated from every genotype. The most plantlets were produced by CY-45 and GK Élet genotypes. Green plantlets were regenerated from seven genotypes while three genotypes produced only albino plantlets (Table 1). Green plant production was higher in case of CY-45 and GK Délibáb than at other varieties.

Discussion

Androgenesis of wheat in isolated microspore culture

Characteristic steps of microspore culture were checked by using CY-45 genotype. Our results are in harmony with the data of previous publications in wheat (Mejza et al. 1993; Indrianto et al. 2001). Osmotic pre-treatment of donor anthers and ideal microspore stage (late uni-nucleate and early bi-nucleate) are very important to successful culture (Reynolds 1984). Ovary co-culture was necessary to production of a lot of embryoids (Mejza et al. 1993; Zheng et al. 2002) because ovaries extracted some chemical substances which protected the developing structures (Letarte et al. 2006).

In our experiments the gametofitic pathway of wheat microspores was changed to sporophitic and developed via direct embryogenesis (Zheng et al. 2003). This morphogenesis pathway in wheat was observed by Indrianto et al. (2001) too. Large numbers of pro-embryos appeared in cultures after one month but green plant regeneration rate was to low. After the increase of green plant production, this technique will open up new approach in genetic transformation of haploid cells and tissues (Folling and Olsen 2001).

Microspore culture of Hungarian cultivars

Androgenesis and in vitro embryogenesis was successfully induced in isolated microspore culture of all varieties. Numerous embryos were developed in cultures and green plants were regenerated from seven genotypes. On the one hand, significant differences were found among genotypes in embryo production and plant regeneration. On the other hand, similar results were achieved by ten genotypes. Microspore culture was usable method in case of wide range of our va-

Table 1. Androgenesis of Hungarian varieties in isolated microspore culture. Different letters show the significant differences ($P<0.05$).

Genotype	Number of ELS/ Petri Dish	Number of plantlets/ Petri Dish	Albino plantlets/ Petri Dish	Green plantlets/ Petri Dish
CY-45	413.5 a	117 a	99.25 a	17.75 a
GK Mini Manó	176.75 c	46.75 b	46.5 b	0.25 c
GK Garaboly	100.25 d	20.25 c	15.75 c	4.5 c
GK Hargita	88.75 d	21.25 c	20,75 c	0.75 c
GK Csongrád	224 bc	27 bc	24.25 bc	2.75 c
GK Délibáb	98.75 d	30.25 bc	18.25 c	12 b
GK Élet	289.25 b	117.75 a	117 a	0.75 c
GK Kata	65 d	3 c	3 c	0 c
GK Bán	31.75 d	8 c	8 c	0 c
Mv Palotás	72.75 d	4.5 c	4.5 c	0 c
$SzD_{5\%}$=	69.98	23.1	23.1	5.15

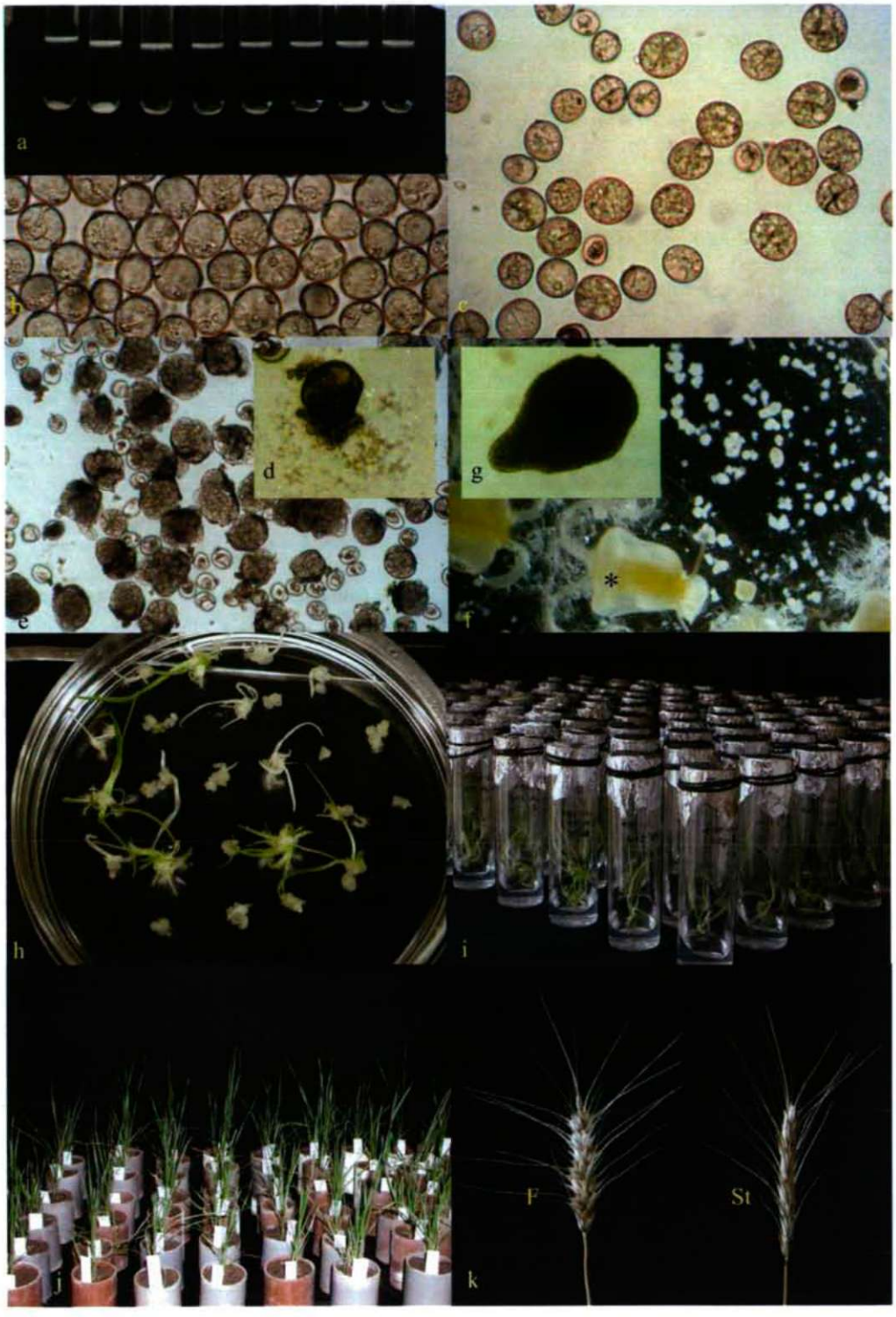

Figure 1. Characteristic steps of microspore-plant system of wheat microspore culture: a, The collected viable microspores in white bands between two solutions after gradient centrifugation (0.3 M mannit, 21% maltose), b, the ideal (late uni- and early binucleate) stage of wheat microspores after isolation, c, Microspores with sistercells at 7th day of microspore culture. d, 10 day-old multi-cellular structures broke out the wall of microspore. e, 14 day-old multi-cellular structures grew intensively, f, 21 day-old pro-embryoids in microspore culture with wheat ovary (*) co-culture, g, microspore-derived wheat embryo. h, Green and albino plantlets were regenerated on regeneration medium. i Microspore culture-derived green plantlets for individual culturing in glass tubes, j, green plantlets were acclimatized in greenhouse. k, Fertile (F) and sterile (St) wheat spikes were obtained on transplanted microspore-derived plants in greenhouse.

rieties. The green plant regeneration rate has to increase for plant breeding. After optimization of culture conditions, this method will be beneficial technique in our wheat breeding programme like anther culture (Szakács et al. 2002).

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ARTICLE

Change of root and rhizosphere characters of willow (*Salix* sp) induced by high heavy metal pollution

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ABSTRACT The abandoned Pb/Zn mine causes heavy metal problems in the surrounding area at Gyöngyösoroszi (North-Hungary). The Toka-river transported heavy metal (Cd, Cu, Pb, Zn) ions from several heaps deposited imprudently near a historic lead and zinc-mining site. Willow (*Salix* sp.) is one of the plants often applied for phytoremediation practice, since its high metal accumulation capacity. Proposal of this work was to investigate the change of root and rhizosphere characters of willow plants induced by high heavy metal pollution. The response of root mass/soil samples, fine root mass/other roots and BAF-s (bioaccumulation factors) and also two soil biological properties soil microbial biomass and acid phosphatase activity to heavy metal pollution were studied. All measured biological parameters have proved to indicate soil pollution, root mass decreased, portion of fine root increased, BAF-s values with exception of Cd decreased significantly. Both measured biological parameters of willow rhizosphere therefore could indicate soil pollution, but change was adverse, biomass decreased while phosphatase activity increased. Microbial biomass and phosphatase activity were not correlated, indicating different account of ecological factors that altering biological properties of a soil.
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KEY WORDS

willow
root
rhizosphere
heavy metal pollution

The mining activity surrounding the historic Pb/Zn mine at Gyöngyösoroszi (Hungary) causes heavy metal problems on the environment through the contamination caused by deposition from stream (Horvath and Gruiz 1996). Element content of plants depends partly on the inherent properties of plants the species, developmental stage and plant part. Willow is one of the plants that often investigated for phytoremediation purposes, having high metal accumulation capacity and easily cultivable characters (Tremela et al. 1997; Pulford and Watson 2003). *Salix* species were reported as Zn-accumulators (Vashegyi et al. 2005), or Cd- and Zn-accumulators (Vandecasteelea et al. 2002; Máthé-Gáspár and Anton 2005). Willow plant is considered that may be the best indicator of elevated Zn and Cd (Pugh et al. 2002).

It is well known that heavy metals causes significant decrease in fresh and dry weight and length of root. Wilkins (1957) and Jowett (1964) introduced a tolerance index (TI), which is a ratio of treated root length to control root length.

Biological and biochemical parameters of the rhizosphere, such as microbial biomass and enzyme activities are considered as indicator of soil quality (Brookes 1995; Szili-Kovács et al. 1998; Simon and Biró 2005; Takács et al. 2005). There are increasing evidences of microbial biomass decrease due to metal contamination in soils (Brookes et al. 1986; Filip 1998). Phosphatase activity showed a sensitive response to metal contamination according to several studies (Máthé and Kovács 1980; Anton et al. 1994; Máthéné Gáspár et al. 2005).

Most investigations are based on the soil metal enrichment in laboratory and very few attempts were made to study it in the field conditions. To study this latter situation is difficult because of the spatial variability of sites and pollutants and it is also critical to find an appropriate non-contaminated control site (Kádár and Németh 2003; Máthé-Gáspár et al. 2004). Szili-Kovács et al. (1999) have investigated experimentally metal polluted field plots seven years after metal loading. All investigated (Cu, Ni, Zn and Cd) polluted soils had lower microbial biomass than the control.

The objective of this paper was to study the change of several root characters, microbial biomass C and phosphomonoesterase activity of willow rhizosphere influencing heavy metal pollution in the field conditions.

Materials and Methods

Experimental site

Experimental site is located at the bank of Toka-river near Gyöngyösoroszi village (North-East Hungary) near an abandoned Pb/Zn mine. The soil type is Fluvisol. The climate of the region is temperate with continental features. The vegetation is heterogeneous containing natural and weedy elements

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Table 1. Main metal accumulation in the topsoil at the polluted and unpolluted experimental sites along the Toka river. Different letter in upper index of the corresponding parameters means significant difference at $p<0.05$.

Element content, mg kg^{-1} soil									
Unpolluted soil					Polluted soil				
samples	Cd	Cu	Pb	Zn	samples	Cd	Cu	Pb	Zn
1	0.360	62.3	35	141	1	19.7	325	1409	3181
2	0.618	84.5	47	211	2	28.8	493	2827	4417
3	0.932	100	65	274	3	11.9	197	724	2190
4	1.05	90.4	64	285	4	18.9	298	1183	3185
5	0.575	87.5	47	199	5	16.9	315	1620	2873
6	0.698	105	56	221	6	15.4	364	1974	2650
mean	0.71^a	88^a	52^a	222^a	mean	18.6^b	332^b	1623^b	3083^b

as well. A phytoremediation experiment was set up in 2003 year by planting willow trees in rows along the contaminated and uncontaminated sites. The contaminated area is located approximately a 10-metre-wide strip along the river. The site at higher distance from the river is considered to be unpolluted according to the soil chemical data.

Plant and soil sampling and chemical analyses

Plant and rhizosphere soil samples were taken at six unpolluted (UP) and six polluted (P) points around planted willows (*Salix* sp.) in the 6th of October 2004 year from 0-20 cm depth. The moist samples were sieved (2 mm mesh) and stored at 4°C until the analyses has been performed. Root and soil samples were separated. Root samples after mechanical cleaning and washed with deionized water, and then dried at 70°C until the stabilization of weight, soil samples were dried at 105°C.

The metal concentrations of soils and plant roots were determined after standard preparation (soil extraction by HCl/HNO_3 and NH_4-acetate –EDTA and plant by HNO_3) by ICP spectrometry. Basic soil properties like humus content, pH, $CaCO_3$, plasticity index (K_A), salt, moisture content was measured.

Microbial biomass C and phosphatase activity

Microbial biomass C of the rhizosphere soil samples of willow was estimated by chloroform fumigation extraction (Vance et al. 1987). Fifteen g of soil was fumigated by chloroform in a desiccator for 2 min then left it overnight. After chloroform removal by repeated vacuum fumigated and unfumigated samples were extracted by 0.1 M K_2SO_4 after shaking filtered and the organic carbon was measured by a combustion TOC analyzer (Shimadzu). Biomass C was calculated as the difference of extracted organic C between the fumigated and unfumigated samples multiplied by a conversion factor, k_{EC} = 2.63 proposed by Vance et al. (1987).

Acid phosphatase activities were determined in the sampling time according to Tabatabai and Bremner (1969). One g of moist fresh soil was incubated in 4 mL modified universal buffer (pH 5.5 for acid phosphatase) and 1mL p-nitrophenyl phosphate (15 mM) for 1h at 37°C. After incubation, 1 mL $CaCl_2$ (0.5 M) and 4 mL NaOH (0.5 M) were added to stop the reaction and to increase the pH. The nitrophenol concentration was determined photometrically at 410 nm.

Data analysis

Root mass characters, chemical data of roots and soil samples, the biomass C and acid phosphomonoesterase activity values between the polluted and unpolluted sites were compared by two-samples *t*-test.

Results and Discussion

Heavy metal pollution of soil

Polluted soil samples were characterized by high content of Cd, Cu, Pb, Zn, (11.9-29.8, 197-493, 724-2827, 2190-4417 mg/kg, respectively (Table 1). The repeated flooding may deposit sediments containing metals in elevated concentration. The humus content and pH was not differed significantly between the polluted and unpolluted sites.

Changes of root characters

Heavy metal pollution induced a decrease in the root mass/soil mass value (in the sample mean), but increased in fine root mass/other root mass value (Table 2). Heavy metals in enhanced concentration caused a reduction in root mass (35.54%) but increased fine root mass (468%). Changes indicate significance in differences of growth affected by high heavy metal concentration of soil.

While the concentration of heavy metals has increased in the soil, their amounts in plant roots have decreased therefore the bioaccumulation factors were also decreased (Table 3). Values of BAF of willow root decreased, with the exception of Cd resulting 2.5, in the order of Zn (to 75.8%), Cu (to 66.4%) and Pb (to 38.9%).

Table 2. Root mass/soil mass, fine root mass/other root mass (g dry weight/ g dry weight, %). Different letter in upper index of the corresponding parameters means significant difference at $p<0.05$.

Unpolluted soil samples	root/soil, %	fine root/other root, %	Polluted soil samples	root/soil, %	fine root/other root, %
1	17.60	0.45	1	7.70	1.20
2	11.50	0.62	2	4.72	2.55
3	17.02	0.10	3	6.20	0.96
4	24.00	0.05	4	5.25	1.85
5	16.22	0.58	5	2.34	2.20
6	15.76	0.26	6	11.10	0.89
mean	17.02[a]	0.34[a]	mean	6.22[b]	1.61[b]

Table 3. Bioaccumulation Factors of willow root.

Bioaccumulation Factors Unpolluted soil samples	Cd	Cu	Pb	Zn	Polluted soil samples	Cd	Cu	Pb	Zn
1	4.363	0.14	0.0092	0.9434	1	2.2386	0.042	0.0029	0.4041
2	2.199	0.128	0.0068	0.5806	2	1.5346	0.039	0.0024	0.3672
3	2.134	0.111	0.0155	0.6477	3	3.2484	0.121	0.0067	0.7307
4	2.3163	0.104	-	0.6232	4	2.6224	0.089	0.0038	0.4439
5	2.5001	0.103	0.0121	0.6499	5	3.5803	0.119	0.004	0.667
6	2.5957	0.106	-	0.6944	6	2.8196	0.081	0.0068	0.5915
mean	2.5041	0.114	0.0106	0.6713	mean	2.513	0.075	0.0041	0.5085

Table 4. Mean values of phosphorus (total, LE-soluble), water content, phosphatase activity and microbiological biomass of the rhizosphere soil samples. Different letter in upper index of the corresponding parameters means significant difference at $p<0.05$.

Soil samples	'Total' P content mg kg^{-1} d soil	'LE –P content mg kg^{-1} d soil	Soil water content, %	Phosphatase activity, µmol pNP g^{-1} d soil h^{-1}	Biomass C µg g^{-1} d soil
UP	1112[a]	653[a]	18.74[a]	0.8482[a]	186.2[a]
P	783[a]	71[b]	20.66[b]	1.3242[b]	71.3[b]

Rhizosphere response. Microbial biomass and phosphomonoesterase activity

Microbial biomass C was significantly higher in unpolluted soils comparing with polluted ones (Table 4). The standard errors were higher in unpolluted soils, which might be attributed to the heterogeneous nature of the sample having microsites with various microbial activities. The average of soil microbial biomass C was 186 mg kg^{-1} and 71 mg kg^{-1} for unpolluted and polluted soils respectively.

Phosphomonoesterase activity of unpolluted (UP) soil samples ranged between 0.78 and 0.97 µmol pNP g^{-1} dry soil h^{-1}. A significant increase of acid phosphomonoesterase activity (0.88 and 1.58 µmol pNP g^{-1} dry soil h^{-1}) was determined in the soil due to the pollution. Phosphatase production of living organisms could be stimulated by the higher moisture content and by the significantly lower LE-soluble phosphorus content of the polluted soil samples (Table 4).

All measured biological and biochemical parameters therefore could indicate soil pollution, root mass and microbial biomass decreased, portion of fine root and phosphatase activity increased, BAF-s values with exception of Cd decreased significantly. Changes in observed parameters indicate not only the influence of heavy metals, but also other ecological factors.

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8th International Conference on Membrane Redox Systems and Their Role in Biological Stress and Disease

4-8 April, 2006
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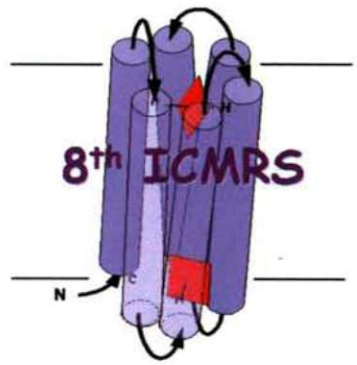

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PROCEEDINGS

Guest Editor: Alajos Bérczi

PREFACE

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What has happend before the 8th ICMRS?

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Twenty years ago, in 1986 there was a scientific cooperation meeting between the National Science Foundation of the USA and the Spanish Consejo Superior De Investigaciones Cientificas (12-14 May 1986, Madrid, Spain). In those days one of the most exciting topics in biology was the possible existence of membrane energizing system(s) other than H^+-transporting and ATP-utilizing trans-membrane proteins (the ATPases) in membranes different from the mitochondrial and chloroplasts membranes. Some of the participants decided to initiate the organization of an international conference on a newly-emerging and quickly-spreading scientific question: What is the physiological significance of the plasma membrane redox system(s), and what is its (their) biochemical nature? The first meeting – entitled "Plasmalemma redox functions in plants" – was organized as a Special Interest Group Meeting (SIGM-08) in the frame of the XIV International Botanical Congress (24 July – 1 August 1987, Berlin-West). The meeting was very successful but had one drawback; only scientists working with plants were participating. Thus participants in Berlin-West decided that an international conference should be organized where scientists dealing with plasma membrane (or cell membrane) redox proteins from any kind of biological objects could meet and discuss the problems common in this research field. The second meeting – entitled "Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth" – was organized as a NATO Advanced Research Workshop (21-25 March 1988, Cordoba, Spain). This was really the very first meeting in which plasma membrane redox systems from very different biological organisms were presented and discussed. Although the next (third) Plasma Membrane Redox Meeting – entitled "Molecular Biology and Function of Plasma Membrane Redox" – was organized only 6 years later (22-25 March 1994, Cordoba, Spain), one had to wait for 10 years after the first Cordoba meeting until the Plasma Membrane Redox Meetings became a regular event. Ever since the 1998 meeting in Antwerp (Belgium) the Plasma Membrane Redox Meetings have maintained the title: "International Conference on Plasma Membrane Redox Systems and Their Role in Biological Stress and Disease". However, the last (the 8th) meeting organized in the Biological Research Center of the Hungarian Academy of Sciences in Szeged, Hungary, on 4-8 April 2006 changed a little the title again for the "8th International Conference on Membrane Redox Systems and Their Role in Biological Stress and Disease" (8th ICMRS-2006: http://www.membranredox2006.hu/).

It is clear from the above introduction that both the title and the scope of the conferences have previously changed from time to time. These changes reflected the shift of interests in the field of research in membrane redox systems. The identification of membrane-associated redox systems in membranes other than the 'energetic' membranes (mitochondria and chloroplasts), started with the observation that perfused liver was capable of reducing the non-permeable electron acceptor ferricyanide (FeCN). These experiments suggested that the cell membrane should also have a redox system. Demonstration of the FeCN-reducing capability of plasma membranes from various plants and animals proved in a very short time that the presence of a redox system in plasma membranes is a ubiquitous event in nature. In the last few years, however, it has become evident that not only the plasma membrane but also other intracellular membranes different from mitochondria and chloroplasts (e.g. the vacuolar membrane, and the membranes of the ER) seem to contain redox system(s) (redox proteins) capable of either cis- or trans-membrane electron transport. The aim of the 8th ICMRS-2006 in Szeged (Hungary) was to provide possibility and a friendly atmosphere for scientist dealing with membrane redox proteins in any cellular membrane preferentially different from the mitochondrial and chloroplast ones. The minor change in the conference title from "Plasma Membrane Redox Systems" to "Membrane Redox Systems" reflects the significant development in the research field of membrane redox systems.

There were two lectures held by 'honorary fathers' of the research field of "plasma membrane redox processes" in the Opening Session. Hans Löw (Stockholm, Sweden) and Michael Böttger (Hamburg, Germany) recalled the beginnings and introduced the audience to the problem of presence and possible function(s) of plasma membrane redox system(s) both in animal and plant tissues. During the next 4 days there were 6 sessions organized with 25 oral presentations and 23 posters in well-defined subjects:

- Membrane-bound Two-heme-containing Proteins (e.g. NOX proteins, proteins belonging to the cytochrome b561

protein family)

- Membrane-bound Redox Proteins (e.g. nitrate reductases, quinone reductases, flavoenzymes)

- Plasma Membrane Redox Proteins (e.g. peroxidases, disulfide isomerases, membrane-bound c-type cytochromes)

- Heavy Metals and Membrane-bound Redox Proteins (e.g. Zn-, Cu-, Fe-containing proteins, problems of heavy metal acquisition)

- Quinones in Membrane Redox Processes (e.g. Vitamins K, ubiquinone, Q10)

- Stress and Disease (e.g. membrane redox processes in relation to Alzheimer's disease, Parkinson's disease, cancer, atherosclerosis, phytoremediation, disorders in heavy metal acquisition, etc.)

Each session had one or two plenary lectures with invited speakers and two or three oral presentations. While lectures in the first 5 sessions listed above were connected to basic research, the 'Stress and Disease' session provided possibility for researchers to present their new results in the fields where involvement of membrane redox processes are known to be important in diseases. Since all posters were on show next to the lecture hall and throughout the conference, there was almost unlimited possibility and time for discussions even beyond the official Poster Session.

In the present issue of Acta Biologica Szegediensis, 9 proceedings are published which represent a fair cross-section of scientific subjects dealt by the 57 participants from 4 continents of the Globe. Only correction in English was made at some places, however, special care was taken not to alter the content that remained the author's full responsibility.

I hereby express my thanks to my colleagues for helping in organizing the 8th ICMRS in Szeged, Hungary. Special thanks are to Drs. Balázs Szalontai and Zoltán Kóta for their expertise in preparation of the Abstract Book and the web site of the Conference, and to The Scientific Organizing Committee for helping in organizing the sessions. The Conference was generously sponsored by a grant from the Agency for Research Fund Management and Research Exploitation (KPI) in Hungary (OMFB-00622/2006).

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Role of domain interactions during the amyloid formation of yeast phosphoglycerate kinase

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ABSTRACT Beta-amyloids are known to be the cause of an increased oxidative stress, which manifests in a higher rate of membrane lipid oxidation in some diseases. There are several proteins that are built up of two structural domains and are deposited full-length in amyloid plaques formed during different diseases. Several publications prove the role of the domain-domain interactions in protein folding, but the effect of the domain interactions on misfolding and amyloid formation has not been tested yet. In this work we show the importance of the inter-domain interactions in amyloid formation. A model protein system based on mutants of the two-domain protein yeast phosphoglycerate kinase was used to study the role of domain interactions in the amyloid formation of multi-domain proteins. After the initiation of the amyloid formation, tryptophan fluorescence spectroscopy was used to detect the structural changes of the two domains from 5 minutes to 4 days. We compared the kinetics of amyloid formation of the individual domains with that of the intact protein. For all mutants, electron micrographs proved the formation of amyloid fibrils after 5 days. We found that the aggregation-coupled conformation changes of the two domains are synchronized in the protein through the domain-domain interactions.

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KEY WORDS

amyloid formation
domain interactions
tryptophan fluorescence
phosphoglycerate kinase
misfolding

An important general property of all proteins is that they can fold into their enzymatically active structure. The three-dimensional structural information is coded linearly in the amino acid sequence of the protein. The formation of the native state is guided by non-local interactions between the amino acid side chains. Under stress conditions non-native structures can also form. These misfolded proteins are often prone to aggregate and under certain circumstances instead of being repaired or cleaned up with the help of chaperones and proteases, they can accumulate into protease resistant fibrils or plaques called amyloids. Beta-amyloids were found to be the cause of an increased oxidative stress, which manifests in a higher rate of membrane lipid oxidation in some diseases (Nelson and Alkon 2005). The deposition of amyloid plaques was observed in several diseases (e.g. Creutzfeldt-Jacob disease, Alzheimer disease, Parkinson disease, type II diabetes), and it can lead to tissue degradation and loss of organ function (Serpell et al. 1997). Only a small number of proteins was found to form amyloid plaques *in vivo*. Some of these formed amyloids *in vitro* as well (Dobson 2001). Recently, several non-pathogenic proteins were found to be able to grow amyloids *in vitro* under conditions that strongly destabilize the native structure and favor hydrogen bonding (Fädrich et al. 2001). It has been suggested that the ability to form amyloids under appropriate conditions is a property common to many if not all proteins. Amyloid structures are stabilized by hydrogen bonding between the atoms of the polypeptide backbone as opposed to the interactions between the amino acid side chains stabilizing the native state, thus amyloid formation reflects a polymer nature of the proteins. Some of the proteins observed to be deposited full length *in vivo* in amyloid plaques contain two differently structured and interacting domains. The prion protein, connected to the Creutzfeldt-Jacob disease consists of a structured domain and a second one apparently lacking well-ordered structure. The lysosome deposited in familial amyloidosis and the Ure2 yeast prion protein are both comprised of two interacting structured domains.

Damaschun et al. (2000) have shown that the non-disease-related yeast phosphoglycerate kinase (PGK) is able to form amyloid fibrils *in vitro*. The presence of amyloids was proved by electron microscopy and X-ray scattering a few days after the addition of 190 mM NaCl to the protein denaturated in 10 mM HCl.

Yeast PGK is a 415 residue large monomeric protein. The native state is built up of two domains about equivalent in size, linked by a helical hinge. It has a combined total of 15 α-helices and 15 β-strands. The structure of the domains is similar, consisting of a core of a six-stranded parallel β-sheet that is surrounded by a series of helices, in addition, the C terminal domain has three shorter β-strands. Far UV circular dichroism measurements indicated that this structure is replaced during misfolding and amyloid formation with a fold

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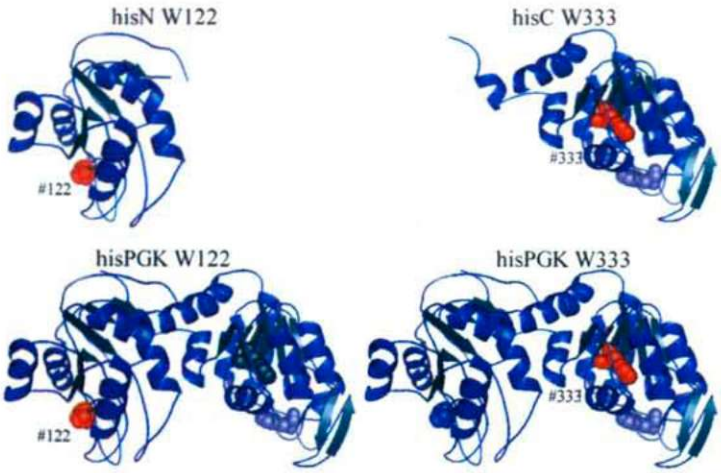

PGK WT	hisN W122	hisPGK W122	hisC W333	hisPGK W333
full sequence	*residues 1-185*	full sequence	*residues 186-415*	full sequence
No his-tag	*his-tagged*	*his-tagged*	*his-tagged*	*his-tagged*
Y122	*W122*	*W122*		Y122
W308		*F308*	*F308*	*F308*
W333		*F333*	*F333*	W333

Figure 1. 3D representation of the PGK variants used in our model protein system (Watson et al. 1982). The location where the single tryptophan reporters were introduced is indicated in red. Constructing the model system involved the mutation of the residues Trp 308, Trp 333, and Tyr 122, these are shown as "spacefill". Alpha helices are colored blue, and beta sheets green. Sequence differences between the wild type yeast PGK and the mutants are listed in italics in the table.

that has significantly higher β-sheet content. Enrichment of β-sheet structure is a general feature of amyloid growth.

Several studies address the role of domain stability and inter-domain contacts in the folding reaction. Yeast PGK has been used successfully as model for the investigation of domain interactions (Beechem et al. 1995). Despite of its possible importance, the effect of the domain interactions on misfolding and amyloid formation has not been tested yet. Here we show that contacts between the two domains of PGK have a strong effect on the misfolding pathway during amyloid formation. These also influence the details of the final fibrillar structure formed.

Materials and Methods

Mutants of the histidine tagged variant of yeast PGK (hisPGK), the histidine tagged variant of the N terminal domain (1-186) of yeast PGK (hisN) and the histidine tagged variant of the C terminal domain (187-412) of yeast PGK (hisC) were constructed, expressed, purified and stored as described earlier (Osváth et al. 2003).

The mutants were acid unfolded by dialysis against 10 mM HCl prior to the experiment. Misfolding was initiated by the addition of 200 mM NaCl to the acid unfolded (10 mM HCl) protein. Kinetic changes were recorded using tryptophan fluorescence from 5 minutes to 4 days. Fluorescence spectra and manual mixing experiments were measured on an Edinburgh Analytical Instruments C-900 luminometer (Edinburgh, Scotland, UK).

For every point of the kinetics, the fluorescence emission spectrum was recorded from 300 nm to 400 nm with 0.5 nm steps. Fluorescence was excited at 295 nm with 5 nm spectral width while detection bandwidth was 1 nm.

Results

A model system (Fig. 1) comprised of two mutant pairs of single tryptophan mutants of yeast PGK was used to investigate the importance of the domain interactions in amyloid formation. The model system comprises single tryptophan mutants of the isolated N and C domains and the corresponding mutants of the intact protein. Fluorescence measurements allowed the comparison of the structural changes in the vicinity of the tryptophan residues of the isolated domains and that of the whole protein during the amyloid formation.

Misfolding was initiated by adding 200 mM NaCl to the protein unfolded in 10 mM HCl. Following this, amyloids grew from all variants of the yeast PGK studied in this work. The presence of amyloid fibrils was checked after 5 days using electron microscopy. The amyloids visualized this way were of roughly identical size for all mutants. Figure 2 shows the spectral shift accompanying the misfolding, aggregation and fibril formation process 17 minutes, 3 hours and 4 days after initiating amyloid formation.

In order to describe quantitatively the time dependence of the shift of the spectrum during amyloid formation, the ratio of the fluorescence intensity integrated between 310-320 nm and 360-370 nm was calculated from the individual spectra.

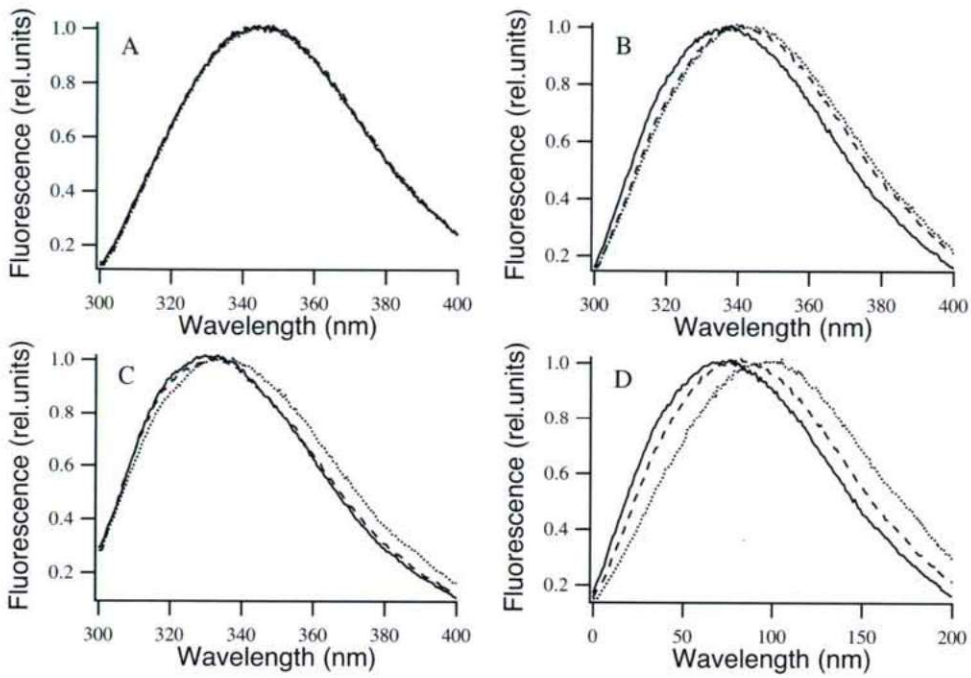

Figure 2. Changes of tryptophan fluorescence emission spectra during the misfolding and amyloid formation of hisN W122 (A), hisC W333 (B), hisPGK W122 (C) and hisPGK W333 (D). Spectra were recorded 17 minutes (dotted line), 3 hours (dashed line) and 4 days (continuous line) after initiating misfolding by the addition of 200 mM NaCl to the samples containing 80 ± 4 μM acid unfolded protein. Tryptophan fluorescence was excited at 295 nm with a spectral width of 5 nm and detected with 1 nm bandwidth.

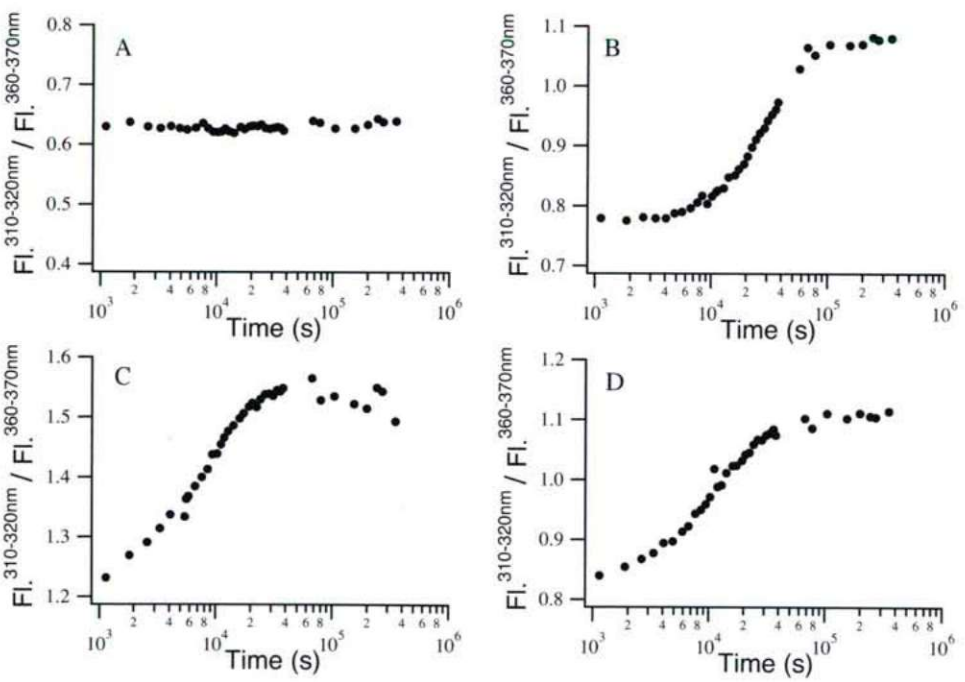

Figure 3. Conformation changes in the four mutants of the model system during amyloid formation. Structural changes were monitored by the blue shift of the fluorescence emission of the single tryptophan residues quantified as the ratio of the intensities emitted in the 310-320 nm and 360-370 nm regions. Fluorescence was excited at 295 nm with 5 nm bandwidth and detected with 1 nm spectral width.

Figure 3 shows the time dependence of the calculated ratio from 5 minutes to 4 days. hisPGK W122, hisC W333 and hisPGK W333 all show a blue shift in the 10^3 to $5 \cdot 10^4$ s region, but hisPGK W122 shows no spectral changes. After $5 \cdot 10^4$ s no further changes were observed in either of the mutants. The kinetics of the spectral change is very different for both domains from the corresponding intact protein, but the tryptophan residues placed at positions 122 and 333 report a similar kinetic course for the misfolding of the complete enzyme.

Discussion

Although there are several diseases in which two-domain proteins are deposited full length in amyloid plaques, the role of domain-domain interactions during misfolding and amyloid formation of proteins has been overlooked. A possible reason for this could be that the domain structure of the native state is destabilized and probably eventually abolished during misfolding and amyloid formation, thus one could expect, that domain interactions bear no importance in amyloid formation. Contrary to this expectation, here we show that contacts between the two unfolded domains of yeast PGK have a strong effect on the amyloid fibril formation. The presence of the complete polypeptide domain also influences the architecture of the final fibrillar structure. This can be of great importance for the *in vivo* scavenging mechanisms, since the resistance to proteases may depend on the molecular details of the formed structure. Our findings can be rationalized if we take into account that the folding pathways of proteins depend strongly on the stability of the folding domains relative to the domain interactions. Since misfolding often occurs from a destabilized native state of the protein or a collapsed partly folded intermediate, the misfolding pathways of the destabilized domains can be easily determined by the domain interactions. This is in perfect agreement with the findings that one structural domain of the human lysozyme deposited in fatal familial insomnia is destabilized in all its amyloidogenic mutants.

Misfolding and amyloid formation of yeast PGK was studied by manual mixing measurements using two single tryptophan mutant pairs designed to compare the misfolding properties of the individual domains and of the domains within the complete protein. Fluorescence emission spectra revealed that the tryptophan labels are more solvent exposed in the intermediate formed 5 minutes after the initiation of the amyloid formation than in the native state, but less exposed than in the acid unfolded state. Our results are in accord with the findings of Modler et al. (2003) who have proved the presence of a partially refolded intermediate at this stage of the yeast PGK misfolding. This partially folded intermediate is the starting point for protein aggregation and amyloid formation. The tryptophan emission spectra measured 4 days after initializing misfolding were found to be different for the individual domains and the corresponding complete proteins, thus the final structure adopted within the amyloid depends sensitively on the presence of the other domain.

We found that the two individual domains show different misfolding kinetics, but in the corresponding mutants of the complete protein misfolding proceeds differently from the individual domains, and similarly to each other. This shows that long-range interactions play a decisive effect on the misfolding pathway. All amyloids have a characteristic β-sheet structure regardless of the sequence of the protein. Our results however show that the sequence specific contacts such as the long-range interactions between different parts of the polypeptide chain are of great importance in all steps of the misfolding and amyloid growth. Domain interactions also determine the conformation of the polypeptide chain adopted in the amyloid fibrils by influencing the early misfolding steps.

In conclusion we can say that: Amyloid-like fibrils grew from mutants of the individual domains and the complete protein under the same conditions.

Interactions between the N and C terminal polypeptide regions influenced the amyloid formation and influenced the final tertiary structure formed as well.

Conformational changes were different for the individual domains, but in the complete protein these changes were synchronized by the interactions between the C and N terminal domains of the protein.

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Reactions of root plasma membrane redox activities in iron-deficient cucumber plants after application of ionic and chelated copper

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ABSTRACT The effects of ionic ($CuSO_4$) and chelated forms of copper (Cu(II)HEDTA, where HEDTA is N-(2-hydroxyethyl) ethylenediamine triacetic acid, applied at micromolar concentrations in nutrient solutions of cucumber plants grown hydroponically under conditions of iron deficiency (–Fe), were studied. Changes of plasma membrane reductase activity (PMRA) of intact roots after treatment with ionic or chelated copper were followed in (+Fe) and (–Fe) cucumber plants. Iron deprivation in nutrient solution provoked a great increase of ferric-chelate reductase activity (with substrate of Fe(III)HEDTA) and accelerated the cupric-chelate reductase activity (measured with Cu(II)Citrate as an electron acceptor) as well as the hexacyanoferrate(III) [HCF(III)] reductase activity. Continuous application of cupric ions in solutions of iron-deficient plants resulted in a dramatic inhibition of Fe(III)HEDTA and Cu(II)Citrate reductase activity. The reductase activity in iron-deficient cucumber roots, measured with HCF(III), was inhibited to a lower extent after cupric ions treatment. On the other hand, the cupric-chelate Cu(II)HEDTA, applied at the same concentrations in solutions with (–Fe) plants, maintained the high stimulation of plasma membrane ferric-chelate and cupric-chelate reductase activity and produced additional acceleration of HCF(III) reduction by cucumber roots. The treatment with Cu(II)HEDTA improved the growth and root PMRA as well as other iron-deficiency stress responses of cucumber plants.
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KEY WORDS

cucumber (*Cucumis sativus* L.),
iron deficiency
ionic and chelated copper treatment
ferric- and cupric-chelate reductase activity
hexacyanoferrate(III) reduction

Copper and iron as important micronutrients with similar redox-properties need strict control in their mobilisation, uptake and translocation. The regulation of Fe and Cu homeostasis in plant cells under non-optimal growth conditions is extremely important for both plant productivity and human nutrition. Iron deficiency chlorosis as a limiting factor for plant growth and yield reduction is spread in different crops, mainly in alkaline carbonate soils, due to the insolubility of iron oxides and hydroxides (Schmidt 1999). Additionally, Fe-insufficiency stress can be combined with increased level of copper moving in plant tissues by fungicide spraying against diseases (Babalakova et al. 2003). In conditions of iron deficiency dicotyledonous plants such as cucumber develop various adaptive morphological and biochemical mechanisms to improve iron acquisition in soil solutions (Fe-deficiency stress responses; Raboti et al. 1995; Espen et al. 2000). The main adaptive process includes a strong increase in plasma membrane (PM) ferric-chelate reductase activity by roots accompanied with enhanced proton release needed for the reduction of Fe(III) to more soluble Fe(II) in the apoplast. Acidification of the rhizosphere is fulfilled by activation of PM proton pump and the biosynthesis of specific ferrous transporters at PM is accelerated (Robinson et al.1999; Schmidt 1999; Curie and Briat 2003). The PM-associated ferric-chelate reductase is the most studied redox enzyme; it is an integral membrane protein belonging to a family of flavoproteins that transfer electrons from cytosolic NADH to extracellular electron acceptors via FAD and heme groups (Robinson et al. 1999; Curie and Briat 2003). Besides a high induction of ferric-chelate reductase activity (FeChRA) in roots of iron-deficient plants, the reduction capacity for cupric compounds as well as hexacyanoferrate(III) reductase activity [HCF(III) RA] was also stimulated by iron deficiency, however, their relation to the uptake of copper and iron is not clear (Welch et al.1993; Babalakova and Schmidt 1996; Holden et al.1996; Weger 1999). The enhanced activity of the root PM redox system might contribute to cation uptake alteration under conditions of iron deficiency. Thus comparison of ferric- and cupric-chelate reduction by intact cucumber plants deserved attention. It was established that iron deprivation brought to increased content of copper in roots (Herbik et al.1996), but was also shown that ionic copper produced an inhibition of both induction and function of FeChRA in roots of iron-deficient plants (Alcantara et al.1994; Romera et al.1997; Schmidt et al.1997; Cohen et al.1998). However, it is not clear whether or not cupric chelate affects the regulatory control of the response to Fe-deficiency. In our earlier study we have established that short-term application of chelated copper, Cu(II)HEDTA, sustained the high activity of ferric-chelate reductase in roots of iron-deficient cucumber plants,

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kept the proton release and additionally stimulated cupric-chelate and HCF(III) RA. These effects were opposite to the strong inhibitory action of free copper ions, applied at the same concentrations in solutions of iron-deficient plants (Babalakova et al. 2005). The aim of the present study was (i) to investigate the influence of longer-term application (11-12 days) of Cu(II)HEDTA and free Cu-ions in the nutrient solution of control (+Fe) and Fe-deficient (–Fe) cucumber plants, (ii) to follow the growth and reactions of plasma membrane ferric-and cupric-chelate RA in roots of intact cucumber plants, and (iii) to study the activity of standard PM redox system (with HCF(III) as electron acceptor) in control and iron-deficient plants.

Materials and Methods

Plant material

Seeds of cucumber (*Cucumis sativus* L. cv. Gergana) were germinated in Petri dishes on filter paper moistened with 0.1 mM $CaCl_2$ in the dark at 28°C for 3 days. After 3 days the seedlings were transferred to grow in Hoagland-Arnon I nutrient solution (pH 6.0) in plastic pots in an environmental chamber. The nutrient solution was changed every second day and supplemented with 20 µM Fe(III)HEDTA [Fe(III) complex of N-(2-hydroxyethyl) ethylenediamine triacetic acid] and 0.2 µM of $CuSO_4$ for control (+Fe) plants (Babalakova et al. 2004). After receiving one-tenth concentration of the nutrient solution, Fe-deficient cucumber plants were grown in such solutions without Fe. Cucumber plants were harvested 12-13 days after the start of treatments, and morphological parameters – root, stem and leaves fresh weight (FW) – were measured and the reductase activities (RA) of roots of intact plants were determined.

Long-term treatment of control and Fe-deficient plants with ionic and chelated copper

Four-day-old seedlings were treated with different concentrations of $CuSO_4$ (0.2, 2, 10 and 20 µM) or Cu(II)HEDTA (0.2, 2, 20 and 100 µM; prepared as a stock solution, pH 6.0, at ratio of Cu to HEDTA of 1:1.25 as Tris-KOH complex). Treatment solutions were changed on every other day for twelve days. Fe(III)HEDTA used for (+Fe) plants was prepared as a stock solution, at ratio of $FeCl_3$ to HEDTA of 1:1.25 as Tris-KOH complex, pH 5.5. The chemical forms of applied copper, used to compare their effects in control (+Fe) and Fe-deficient (–Fe) plants, have different electrical charges. Copper in copper(II) sulfate forms a cupri-hexahydrate cation, $[Cu(II)(OH_2)_6]^{2+}$, in aqueous solution and keeps the ionic properties of copper. When HEDTA is added to aqueous solution of copper(II) sulfate, HEDTA forms a strong chelate with Cu(II), hydroxyethyl ethylenediamine-triacetato cuprate $[Cu(II)HEDTA]^{2-}$, with anionic character (Coombes et al. 1978).

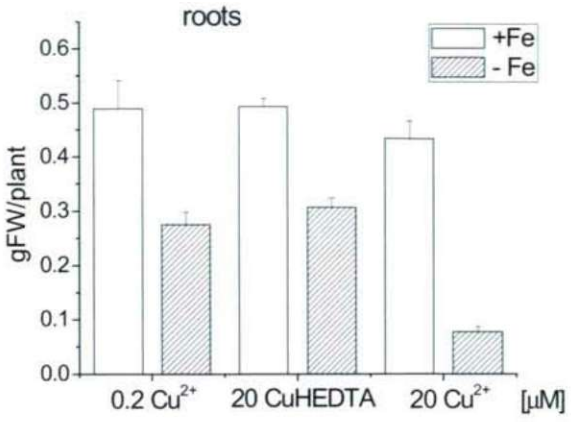

Figure 1. Influence of treatment with ionic copper (0.2 µM and 20 µM Cu^{2+}) or chelated copper (20 µM CuHEDTA) on the root fresh weight of control (+Fe) and Fe-deficient (–Fe) cucumber plants.

Measurement of ferric- and cupric-chelate reductase activity by intact roots

Fe(III)HEDTA and Cu(II)Citrate (as a more natural substrate) were used as electron acceptors. Cupric citrate was prepared at ratio of $CuCl_2$ to Citrate 1:3 as Tris-KOH complex, pH 6.5. The incubation medium for reductase activity measurements contained 0.1 mM $CaCl_2$, 0.15 mM Fe(III) or Cu(II) complex and 0.3 mM BPDS or BCDS in a final volume of 10 or 15 ml in dark vessels at pH 5.5 for Fe(III)ChRA and pH 6.5 for Cu(II)ChRA as described previously (Babalakova and Schmidt 1996; Babalakova and Traykova 2001). The reductase activity of intact roots was expressed in µmol $Fe(II) \cdot g^{-1}$ root $FW \cdot h^{-1}$ or $Cu(I) \cdot g^{-1}$ root $FW \cdot h^{-1}$. HCF(III) RA was performed according to Schmidt (1994).

Statistical analysis

The experiments were repeated at least 3 times with 6 to 8 intact plants in each variant. The data presented are the average of 18 to 24 samples and the values in the figures and tables represent the standard errors of the mean values. Differences between variants were compared by Student's *t*-test at 5% level of significance.

Results

Two-weeks-old control cucumber plants (+Fe; 0.2 µM Cu^{2+}) had expanded green cotyledons and first and second truly green leaves. Cucumber plants grown 11-12 days without Fe in the nutrient solution developed leaf chlorosis. Reduction of the root growth with morphological changes, characteristic of iron deficiency, was also observed. Depending on the chemical form of used copper, the decrease of cucumber root fresh weight (RFW) under conditions of iron starvation was dif-

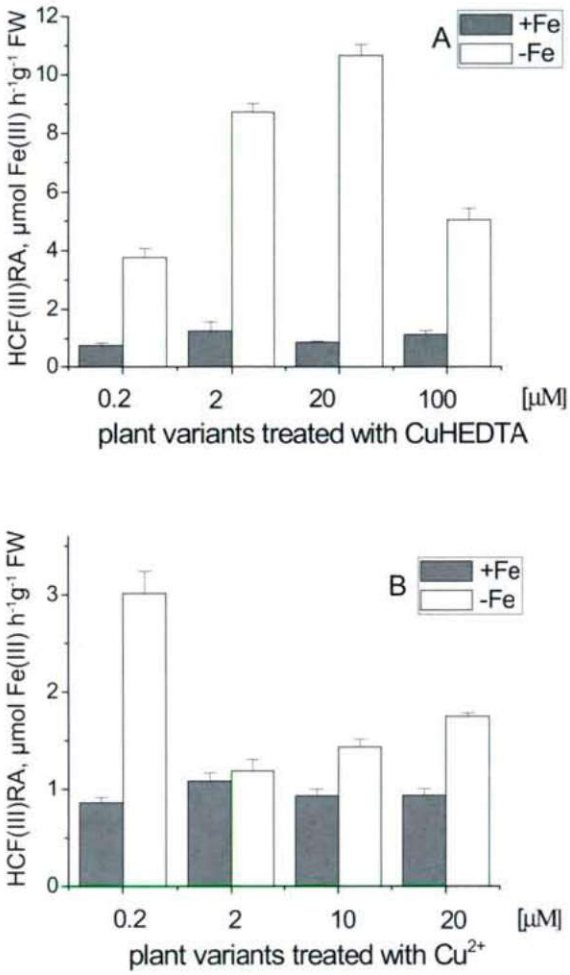

Figure 2. Changes in hexacyanoferrate(III) reductase activity [HCF(III)RA] at root plasma membrane of cucumber plants grown in conditions of normal iron nutrition (+Fe) or under iron deficiency (–Fe), and treated with various concentrations of cupric-chelate (CuHEDTA; A) or free cupric ions (Cu^{2+}; B).

ferent (Fig. 1). Fe-deficiency in solutions containing control level of copper (0.2 µM) provoked about 45-50% reduction of RFW of (–Fe) plants as compared to (+Fe) plants. The application, however, of 20 µM free copper ions for 12 days resulted in dramatic inhibition of the growth and mass production of Fe-deficient cucumber plants. RFW was reduced by about 85% and plants remained small and undeveloped. The same concentration of Cu(II)HEDTA (20µM) markedly improved the growth of entire cucumber plants under conditions of Fe-shortage and remedied RFW of (–Fe) plants about 3.5 to 4 fold during the experimental period (Fig. 1). The continuous treatment with cupric chelate was followed with decrease of leaf chlorosis (data not shown). Micromolar concentrations of copper used (ionic or chelated) did not or only slightly influenced the growth of control (+Fe) plants. We have chosen the concentration of 20 µM cupric chelate to show marked growth improvement of Fe-deficient cucumber plants, as compared to the high inhibitory growth at the same concentration of ionic copper (Fig. 1). On the other hand, (–Fe)-plants treated with 2 µM Cu^{2+} developed very strong chlorosis but growth of leaves was not inhibited, while the growth of plants treated with 20 µM Cu^{2+} were strongly inhibited.

Under conditions of iron deficiency, considerable enhancement of ferric- and cupric-chelate RA was found in cucumber roots (Table 1; about 7- fold increase in (–Fe)-plants). Cupric-chelate reductase activity was measured with Cu(II)Citrate; Cu(II)Citrate is a more natural substrate but chemically it is a weak chelate and more stable at alkaline pH (6.5 was used by us). Added Tris stabilized the complex. However, ferric-chelate reductase activity was measured with Fe(III)HEDTA, a stable chelate at the pH optimum of 5.5. Both reductase activities of intact cucumber roots showed similar increase or decrease depending on the chemical forms of applied copper (Table 1). It was supposed that reduction of ferric- and cupric-chelates by dicotyledonous plants could be performed by the very same membrane reductase (Welch et al.1993). A high inhibition of Cu(II)Citrate RA in (–Fe) plant roots, with respect to their (–Fe) control, was registered after continuous treatment with ionic copper (2 µM and 20 uM). This activity became smaller than the control activity in (+Fe) plants (about 20% and 50% inhibition; Table 1). The Fe(III)HEDTA reductase activity was inhibited to even higher extent than CuChRA by free copper ions (Table 1). Increasing the external copper concentration to 20µM resulted in further decrease of the reduction rate of both reductases. Application of the same concentrations of cupric chelates in solutions of iron-deficient plants, however, brought to considerable stimulation of reductase activity with both electron acceptors. Cu(II)HEDTA applied in nutrient solutions of (–Fe) plants at concentration of 20 µM provoked about 2.2 - 2.4 fold additional activation of cupric- and ferric-chelate RA. The reductase activities in variants of (+Fe) roots were slightly changed after continuous treatment with copper ions or chelate (Table 1).

Standard (or constitutive) redox system activity at PM of cucumber roots (measured usually with HCF(III) as an electron acceptor) reacted to Fe-starvation by 3-fold increase of reduction rate in comparison to control (+Fe) activity (Fig. 2A and 2B). Continuous supply with Cu(II)HEDTA markedly accelerated HCF(III) RA in Fe-deficient cucumber roots, proportionally to the increasing concentrations of cupric chelate from 0.2 to 20 µM (Fig. 2A). Treatment with higher concentrations of Cu(II)HEDTA (100µM) caused less increase in the activity but kept it higher with respect to that of the (–Fe) control plants. The application of free cupric ions (2, 10 or 20 µM) in nutrient solutions of iron-deficient cucumber plants resulted in about 50% inhibition of HCF(III)

Table 1. Alteration of cupric(A)- and ferric(B)-chelate reductase activity in roots of Fe-sufficient (+Fe) and Fe-deficient (–Fe) cucumber plants after continuous application of ionic (Cu^{2+}) or chelated (CuCh) copper in the nutrient solution.

VARIANTS (A)	Cu(II)Citrate RA [μmol Cu(I) . g^{-1} FW . h^{-1}]		% -Fe/+Fe
	+Fe	- Fe	
0.2 μM Cu^{2+}	0.158 ± 0.020	1.208 ± 0.093	765
0.2 μM CuCh	0.165 ± 0.019	1.464 ± 0.101	887
2 μM Cu^{2+}	0.174 ± 0.016	0.138 ± 0.006	79
2 μM CuCh	0.181 ± 0.019	1.693 ± 0.071	935
20 μM Cu^{2+}	0.188 ± 0.020	0.102 ± 0.011	54
20 μM CuCh	0.130 ± 0.015	2.204 ± 0.138	1695

VARIANTS (B)	Fe(III)HEDTA RA [μmol Fe(II). g^{-1} FW . h^{-1}]		% -Fe/+Fe
	+Fe	- Fe	
0.2 μM Cu^{2+}	0.271 ± 0.052	1.982 ± 0.152	731
0.2 μM CuCh	0.275 ± 0.051	2.498 ± 0.125	908
2 μM Cu^{2+}	0.276 ± 0.050	0.153 ± 0.009	55
2 μM CuCh	0.221 ± 0.023	2.786 ± 0.142	1261
20 μM Cu^{2+}	0.285 ± 0.030	0.045 ± 0.001	16
20 μM CuCh	0.175 ± 0.015	3.093 ± 0.165	1767

RA with respect to (–Fe) plants, but kept it at higher values than the activity was in (+Fe) plants (Fig. 2B).

Discussion

Despite intensive research, the role of chelation in the mechanisms of metal uptake, translocation and metabolism in plants is yet not properly understood. The reactivity of copper ions to form stable complexes and to participate in redox reactions at the plasma membrane put forward the conception that copper can displace iron from Fe(III) complexes in nutrient solutions with iron supply (Guinn and Joham 1963; Taylor and Foy 1985). Data describing what might be the plant reactions towards application of cupric-chelates in the absence of iron are not available. Our results showing the improved growth of (–Fe) cucumber plants (Fig. 1) and the ameliorating effect towards leaf chlorosis (data not shown) after the treatment with cupric chelate do not support the suggestion that the primary toxic effect of Cu(II)EDTA could be the induction of iron deficiency in plant leaves (Taylor and Foy 1985). These authors used, however, very high concentrations of cupric chelate. Also, the increase of HCF(III) RA in roots of (–Fe) plants as well as the considerable stimulation of ferric- and cupric- reductase activity (Table 1 and Fig. 2) correlates with remedying growth after application of cupric chelates. These results are in good agreement with the conception that acceleration of PM redox activity can be directly implicated in the regulation of plant growth (Doering et al. 1998).

Some controversial data exist about the extent of uptake of ionic or chelated elements. It has previously been shown that accumulation of copper might be markedly affected by the chemical form of the applied copper, depending on the charge of Cu-complexes. The comparison of the uptake patterns of a positive copper complex with that of an anionic Cu-complex demonstrated that Cu(II)EDTA was accumulated poorly (Coombes et al. 1977, 1978). Recently, the results of Schmidt et al. (1997) have underlined that both ionic Cu and Cu(II)EDTA can be readily transported through the plasmalemma of root cells. The uptake of intact chelate molecules has already been reported for Cu and Pb (Bell et al. 1991; Vassil et al. 1998).

It was supposed for dicotyledonous plants that reduction of ferric- and cupric-chelates could be performed by the very same membrane reductase (Welch et al. 1993). Later investigations were in accordance with the presence of various redox proteins at the plasma membrane of different organisms that can act as cupric- and ferric-chelate reductases (Babalakova and Schmidt 1996; Holden et al. 1996; Weger 1999). The pH optimum was also different for the two reductases. Other results suggested that the ferric-chelate reductase activity, measured at pH 5.5, in Fe-deficient plants (the "turbo" reductase) might be different from the constitutive redox activity measured with HCF(III) as electron acceptor (Babalakova and Schmidt 1996; Holden et al. 1996; Susin et al. 1996). Despite using different substrates and treatments in our experiments, Fe deficiency caused similar changes in the cupric- and ferric reductase activities and demonstrated the presence of redox proteins with similar properties at PM. High activation of cupric reductase in the roots of Fe-deficient plants might be connected to the same extent with increased copper uptake under iron starvation (Herbic et al. 1996). The direct connection between enhanced cupric-chelate reduction and increased copper content in plant roots, however, is not clear. The induction of both FeChRA and HCF(III) RA under iron starvation in different plants varied to different extent because of enzyme heterogeneity (Schmidt 1994; Lynnes et al. 1998).

The considerable inhibitory effect of ionic copper on the plant root reducing capacity after creation of Fe deficiency confirmed previously obtained results (Alcantara et al.1994; Romera et al. 1997; Schmidt et al. 1997). The alteration of RA in Fe-deficient plants was related to pH changes of the nutrient solutions during iron starvation and copper treatment. Application of ionic copper started to inhibit release of protons by roots of Fe-deficient plants from the first day of solution change (Babalakova et al. 2005) and this inhibition correlated with the high inhibition of FeChRA by ionic copper. At the same time chelated copper application stimulated the H^+ extrusion by the roots of Fe-deficient cucumber plants. Enhanced acidification of the medium during iron starvation is important for the induction of and sustaining the high level of FeChRA in many plants, because the enzyme is pH sensitive (Wei et al. 1997; Schmidt 1999). It has recently

been proved that high apoplastic pH depressed FeChRA and restricted the uptake of Fe(II) into the cytosol (Kosegarten et al. 2004). Thus, one of the possible explanations for the high inhibition of ferric-reduction by free copper ions in roots under Fe-deficiency is the inhibition of proton release. Our results showed a correlation between proton release and stimulation of FeChRA under conditions of iron starvation. It was shown in some publications that the rate of cupric reduction was a function of the free Cu^{2+} as an actual substrate for cupric reductase activity (Holden et al. 1996; Weger 1999). Our results demonstrated that cupric-chelate reductase at the plasma membrane of Fe-deficient cucumber roots expressed a higher activity in the presence of chelated copper. Another possible explanation for the inhibitory effect of ionic copper towards FeChRA in iron-deficient roots is based on the assumption that Cu^{2+} might act as a powerful scavenger of the superoxide radical, which was shown to facilitate Fe-chelate reduction at the plasma membrane (Cakmak et al. 1987; Macri et al. 1992). This suggestion is supported by the experiments with in vitro application of copper ions that produced the inhibition of Fe(III)EDTA RA in (–Fe) plants already within the first minutes (Schmidt et al. 1997). Another effect of ionic copper, inhibiting to a higher extent the proton release in (–Fe) cucumber plants, might be the reduced activity of the plasmalemma proton pump (Babalakova and Hager 1994). Ionic copper could also affect Fe nutrition by inhibition of some components or a subunit of the trans-plasma membrane electron transport chain. In the presence of chelating agents, copper ions form chelates that can be taken up by plant roots (Schmidt et al. 1997). These authors showed that at equimolar Fe and Cu levels, *i.e.* in the presence of Cu chelates, induction of the iron-stress response was not inhibited, thus supporting our results for stimulating effects of cupric chelates on the FeChRA in Fe-deficient cucumber plants.

The exact site of copper action remains to be established. In spite of the intensive research, (i) the role of chelation itself, (ii) the role of different metal complexing agents with different charges in the mechanisms of metal uptake by plants, and (iii) the action of metal complexes in induction of RA at membrane level are still poorly understood. The high increase of standard redox system activity (measured as HCF(III) RA) in Fe-deficient cucumber roots upon application of cupric chelates demonstrated strong enhancement of trans-membrane electron transport that might be also connected with sustained activity of the proton pump. Interactions between the H^+-ATPase activity and the redox state of the cytoplasm have been suggested to play an important role in the regulation of electron transport by the standard redox system (Schmidt 1999). On the other hand, we supposed that treatment with copper chelate might induce the biosynthesis of phytocyanines (or cupredoxins), low-molecular electron–transporting proteins in the plasma membrane that contains copper (Nersissian et al. 2001). Cucumber plants are rich of stellacyanine and we hypothesised on a plausible role of cupredoxins in facilitating the transport of electrons to standard redox system in roots that could explain the high increase of reductase activity. What will be the exact explanation for the reaction of iron-deficient plants with increased reductase activity only under influence of chelated copper, needs further research. As the chelated copper loses its charge or ionic properties, the first place of action could be the plasma membrane of root cells. Further investigations are needed to resolve the problems of how ionic and chelated copper acts at membrane level in (+Fe) and (–Fe) cucumber plants as well as how Cu and Fe is metabolised and translocated to shoots.

Acknowledgements

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Characterization of an ascorbate-reducible cytochrome *b*561 by site-directed mutagenesis

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ABSTRACT Ascorbate(ASC)-reducible cytochrome *b*561 (Cyt-*b*561) proteins are present in both plants and animals and create a well-distinguished protein family amongst the two-heme containing *b*-type cytochromes. One isoform of the Cyts-*b*561 identified by genomic analysis of *Arabidopsis thaliana* has been localized in the tonoplast. We have expressed the tonoplast-localized Cyt-*b*561 (TCyt-*b*561) in yeast (*Saccharomyces serevisiae*) cells and shown that the biophysical properties of the recombinant TCyt-*b*561 is very similar to those of the chromaffin granule Cyt-*b*561 (CGCyt-*b*561). Mutation of 4 well-conserved histidine residues (H50, H83, H117, H156) resulted in different expression levels and revealed the importance of these 4 His residues in heme binding and protein expression. Modification of the protein by FLAG-tag or His_6-tag resulted in different degrees of reduced expression levels. When all lysine residues (K70, K76, K79, K80, and K159) in the vicinity of the putative ASC-binding motive were one-by-one replaced by alanine, no major changes in the expression levels were observed. Except in case of the K80A mutant, where the low-affinity ASC-binding constant increased significantly, there were no significant changes in either kinetic parameter characterizing the bi-phase ASC-dependent reduction of TCytb-*b*561. These observations are discussed in comparison to properties of the recombinant CGCyt-*b*561.

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KEY WORDS

ASC reduction
site-directed mutagenesis
cytochrome b561
essential histidine residues
FLAG-tag
His-tag
lysine residues

Ascorbate(ASC)-reducible cytochromes *b*561 (Cyts-*b*561) are a newly described class of intrinsic trans-membrane proteins with two hemes (Wakefield et al. 1986a,b; Asard et al. 2001). Cyts-*b*561 have been identified in a great variety of organisms, including invertebrates, vertebrates, and plants as well (Verelst and Asard, 2003; Tsubaki et al. 2005). All Cyts-*b*561 are predicted to consist of six trans-membrane helices, the central four of them being identified as "Cyt-*b*561 (CB) domain", a structural domain associated with other protein domains in other protein families (Pointing 2001). The CB domain has 4 highly conserved His residues likely involved in coordination of the two hemes (Okuyama et al. 1998). The special location of the four His residues on and two hemes in-between the 4 consecutive trans-membrane helices are one of the common structural characteristics of the Cyt-*b*561 proteins (Bashtovyy et al. 2003).

The only well characterized Cyt-*b*561 protein is the one in chromaffin granule membranes of neuroendocrine tissues, the CGCyt-*b*561. It is believed to catalyze the reduction of intravesicular monodehydroascorbate at the expense of cytoplasmic ASC (Njus et al. 1983; Wakefield et al. 1986a,b; Dhariwal et al. 1991). This electron-transfer reaction replenishes ASC levels inside the granules, supporting the activity of intravesicular monooxygenases such as dopamine β-hydroxylase and peptidyl glycine α-amidating monooxygenase (Kent and Fleming 1987; Seike et al. 2003). Both the natural and the recombinant form of the bovine CGCyt-b561 has been purified to homogeneity (Tsubaki et al. 1997; Liu et al. 2005), however, crystallization and 3-D structure determination of the protein was not yet been achieved.

Four putative Cyt-*b*561 isoforms have been identified first in *Arabidopsis thaliana* (AtCyt-*b*561-1 through AtCyt-*b*561-4) and then in other plants (Asard et al. 2001), however, very little is known about their cellular and/or organ localization and function. One isoform, the AtCyt-*b*561-1 (formerly called CYBASC1 in Griesen et al. 2004), has recently been identified in the tonoplast (Griesen et al. 2004; Shimaoka et al. 2004; Preger et al. 2005), and there is also a speculation of its participation in the iron metabolism of as well as ASC regeneration in the plant cells. Although the tonoplast-localized protein has already been expressed in yeast cells (rAtCyt-*b*561-1; Griesen et al. 2004), the recombinant protein has not yet been purified and charcterized. A tonoplast-localized protein has been purified only partially from beans (Preger et al. 2005) but hardly been characterized yet.

In this paper we show that (1) 4 well-conserved His residues of the recombinant tonoplast-localized AtCyt-*b*561-1 (hereafter called TCyt-*b*561) are essential for heme co-ordinations, (2) FLAG- or His-tag influences the expression levels, and (3) replacing of K80 by Ala increases significantly the low-affinity ASC-binding constant but no other K-to-A replacement influences the ASC reducibility or the kinetics of ASC reduction.

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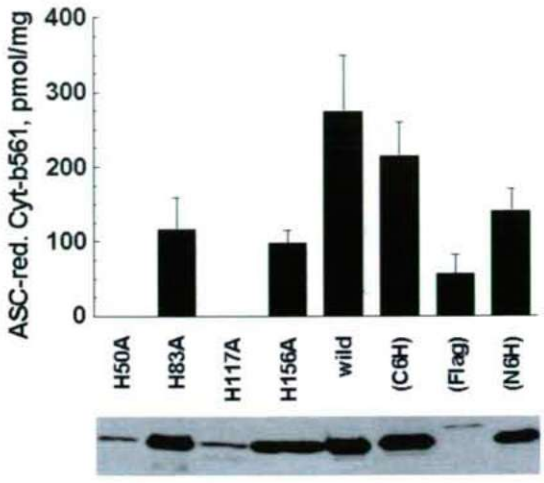

Figure 1. Expression analyses of H-to-A mutant and tagged recombinant TCyt-*b*561: the specific ASC-reduced TCyt-*b*561 content (top part) and by Western blot analyses (bottom part) of the yeast microsomal membranes. Experimental details are in the Materials and Methods. For Western blot analyses, 1 µg of membrane proteins was loaded in lanes for SDS-PAGE. (C6H), His_6-tagged TCyt-*b*561 at its C terminus; (N6H), His_6-tagged TCyt-*b*561 at its N terminus; (Flag), FLAG-tagged TCyt-*b*561 at its C terminus; wild, the recombinant Arabidopsis TCyt-*b*561 without any modification; HxA, H-to-A mutant of TCyt-*b*561 at the "x" position.

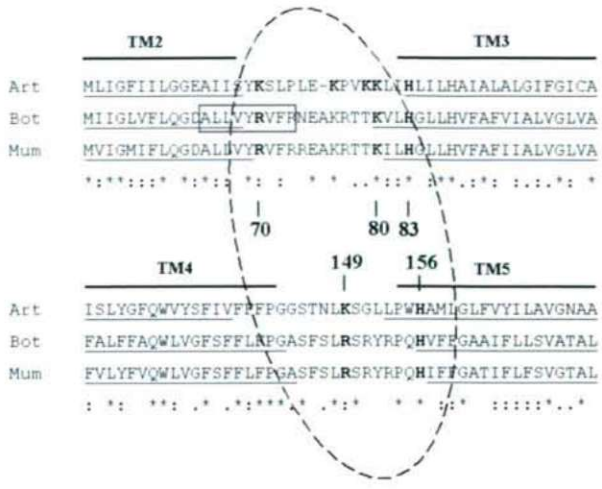

Figure 2. The vicinity (area inside the ellipses) of the ASC-binding motive (boxed area) in the CB domain (Pointing 2001). Partial sequences defining the CB domain are for Arabidopsis (*Arabidopsis thaliana*, Art) TCyt-*b*561, bovine (*Bos taurus*, Bot) CGCyt-*b*561, and mouse (*Mus musculus*, Mum) CGCyt-*b*561. Residue numbering refers to the sequence number in TCyt-*b*561. Bold face letters label residues discussed in the text. TM2 through TM5 label the trans-membrane helices of the CB domain. Underlined sequence regions label the predicted trans-membrane helix of the particular protein.

Materials and Methods

Plasmid construct and yeast transformation

Standard PCR methods were used to amplify the gene encoding AtCyt-*b*561-1 from Arabidopsis mixed tissue total RNA. Primers were designed to include EcoRI and SpeI sites for cloning into the pESC-His expression vector (Stratagene, La Jolla, CA, USA), downstream of the GAL10 Gal-inducible promoter. Sequences were confirmed by DNA sequencing at the University of Nebraska – Lincoln Genomic Core Research Facility.

For the tagged proteins, yeast expression vector pESC-His containing the AtCyt-b561-1 gene (Griesen et al. 2004) was used as a template for PCR reactions to generate N- and C-terminal His-tagged proteins as well as C-terminal FLAG-tagged proteins. Primers were designed as given by Liu et al. (2005) and Griesen at al. (2004), respectively. Standard PCR methods were used to amplify the gene and amplified sequences were confirmed by DNA-sequencing at the University of Nebraska – Lincoln Genomic Core Research Facility.

For transformations, yeast cells (*Saccharomyces serevisiae*, strain YPH499, ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1) were grown in synthetic dextrose (SD) minimal medium (Stratagene, La Jolla, CA, USA) and transformation was performed according to manufacturers instructions. Transformed lines were selected on SD drop-out medium lacking His (SD-His). For induction of protein expression, overnight cultures were grown in SD-His and were transferred to 500 ml of synthetic galactose (SG) minimal medium containing 2% (w/v) galactose and 1 mg/ml sodium ASC.

Antibody production and purification

Antibodies were generated against the 21 amino acid C-terminal polypeptide ([Cys]-SPSPSPSVSNDDSVDFSYSAI) of predicted AtCyt-b561-1 as described before (Griesen et al. 2004). Affinity purified antibodies were used in immunodetections.

Yeast membrane preparation and membrane stripping

Cells from 4 x 450 ml cultures were collected by low-speed centrifugation when the OD_{600} reached a value of 0.9 ± 0.1. Preparation of the microsomal membrane fraction, stripping of membrane vesicles and protein determination were as given by Bérczi et al. (2005). Stripped membrane vesicles were stored in MES-Tris buffer (20 mM MES, 1% (w/v) glycerol, pH adjusted to 7 by addition of Tris salt) at –80°C until use.

Spectroscopy

Absorption spectra of rTCyt-*b*561 in the microsomal membrane vesicles were recorded in dual-wavelength mode (between 500 and 600 nm and reference at 601 nm; OLIS-

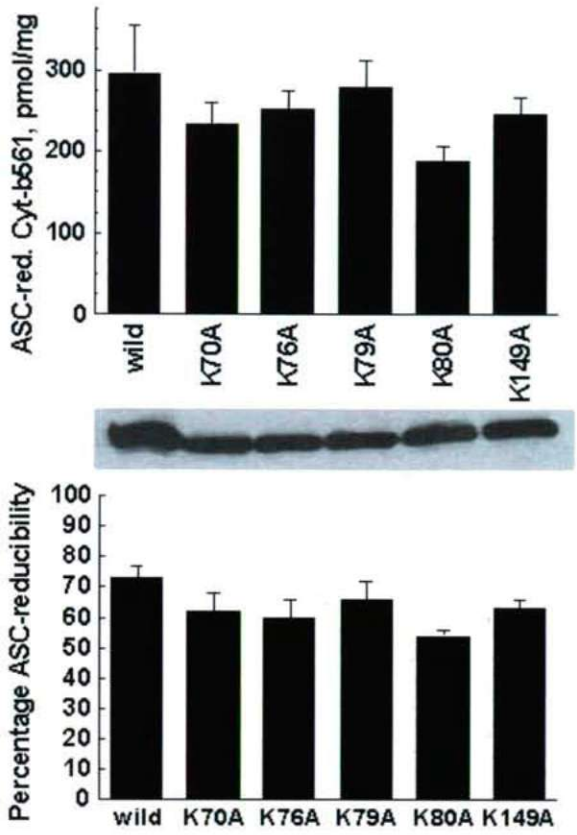

Figure 3. Specific content of K-to-A mutant TCyts-*b*561 in the yeast microsomal membrane fraction (top part), Western blot analyses of the mutants (middle part), and percentage ASC-reducibility of the mutants (bottom part). Experimental details are in the Materials and Methods. For Western blot analyses, 1 μg of membrane proteins was loaded in lanes for SDS-PAGE. KxA, K-to-A mutant of TCyt-*b*561 at the "x" position; wild, the recombinant Arabidopsis TCyt-*b*561 without any modification.

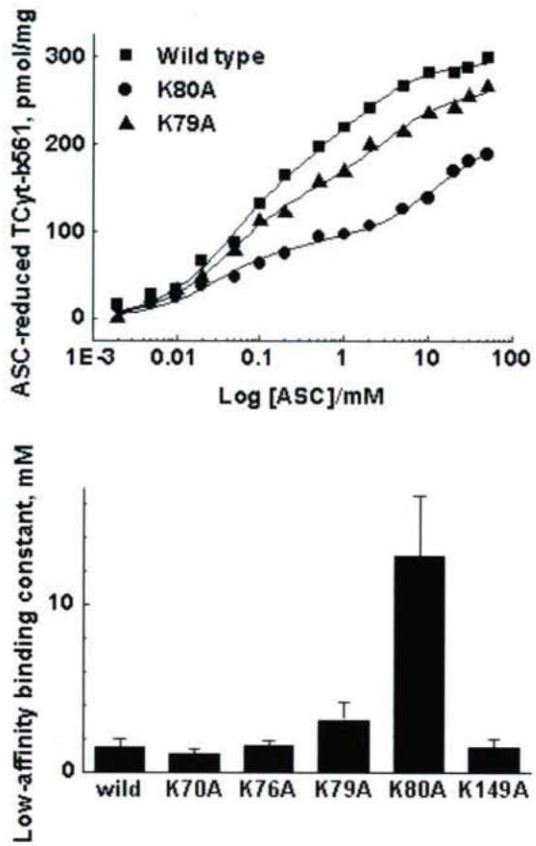

Figure 4. ASC-dependent reduction of TCyt-*b*561 (Wild type) and of K79A and K80A mutants of the wild type protein in the yeast microsomal membrane fraction (top part) and the "low-affinity" ASC-binding constant of TCyt-*b*561 (wild) and of all 5 K-to-A mutants of TCyt-*b*561 (bottom part). Experimental details are in the Materials and Methods. Membrane protein concentration in the optical cuvette was similar for all samples (2 ± 0.2 mg/ml). Top part: experimental points are from one series of experiments. Bottom part: averages with alteration from the average of two independent series of experiments.

updated SLM-Aminco DW2000 spectrophotometer, Bogart, GA) with 1 nm slit-width, 0.5 nm s^{-1} scan rate, and under continuous stirring. Spectra were taken at room temperature in 20 mM MES-Tris buffer, pH 7, 1% (w/v) glycerol, 0.025% (w/v) Triton X-100, 100 mM KCl and in the presence or absence of 0.05 mM ferricyanide or 25 mM ASC or 2.5 mM dithionite. Multiple scans were averaged if improvement of the signal to noise ratio was needed. For calculations, reduced minus oxidized difference spectra and a millimolar extinction coefficient of 30 mM^{-1} cm^{-1} at 561 nm (Tsubaki et al. 1997; Liu et al. 2005) were used. Data analysis and curve fitting was done by using the SPSERV and Origin5.0 softwares. Percentage ASC reduction is the ratio of ASC-reduced to dithionite reduced TCyt-*b*561 multiplied by 100. Data presented on Figs. 1 and 3 are means with standard deviations of at least three independent series of experiments.

Gel electrophoresis and Western blotting

All steps were as detailed by Griesen et al. (2004). Shortly, samples were not heated or boiled prior to loading on the gels, because this caused the proteins recognized by the TCyt-*b*561 antibodies to aggregate, preventing them from penetrating into the gel. Proteins were resolved by SDS-PAGE electrophoresis, using 12% acrylamide gels and transferred onto polyvinylidene difluoride membranes (PVDF, Bio-Rad, Hercules, CA) with a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) in 12 mM Tris, 96 mM glycine, 20% (v/v) methanol. Protein-antibody complexes were detected by horseradish peroxidase-conjugated secondary antibodies (ECL detection kit, Amersham-Pharmacia Biotech, Piscataway, NJ).

Result and Discussion

His mutants and tagged proteins

It has been shown with recombinant mouse CGCyt-*b*561 that the H52-H120 and the H86-H159 pairs of His residues take place in coordination of the two hemes (Bérczi et al. 2005). While the mutation from H to A at positions of H52 and H120 resulted in a loss of protein expression, H-to-A replacement at positions of H86 and H159 resulted only in significant reduction of protein expression. In full agreement with those results, the H50-H117 and the H83-H156 pairs of His residues seem to coordinate the two hemes in TCyt-*b*561 (Fig. 1). While the H-to-A replacement at positions of H50 and H117 resulted in total loss of detectability of TCyt-*b*561, the H-to-A mutation at positions of H83 and H156 resulted only in reduced specific content of TCyt-*b*561 in the microsomal membranes. The spectrophotometric data have been fully supported by Western analyses of the very same membrane fractions for expressed TCyt-*b*561 (Fig. 1). The H83-H156 pair of His residues coordinates the low-potential heme that is located on the cytoplasmic side of TCyt-*b*561 next to a predicted ASC-binding motive (Tsubaki et al. 2000) and assumed to be reduced by ASC.

When TCyt-*b*561 was labeled on its C-terminus by FLAG-tag (Flag) or His_6-tag (C6H) or on its N-terminus by His_6-tag (N6H), the protein expression level hardly decreased with the C6H tag but decreased by 50% and 75% with N6H and Flag tags, respectively (Fig. 1). The Western blot analyses (Fig. 1) supports the spectrophotometric data obtained with the His_6-tagged proteins, but seems to overestimate the decrease in expression of the FLAG-tagged protein. It is very probably that the FLAG-tag at the C terminus of TCyt-*b*561 interferes with the interaction between TCyt-*b*561 and its antibody.

Lysine mutants

Tsubaki et al. (2000) postulated an ASC-binding motive (Fig. 2) and a Lys residue (K85 in the bovine CGCyt-*b*561) as major components in ASC binding and ASC-dependent reduction of CGCyt-*b*561. Recent results with point-mutated recombinant mouse CGCyt-*b*561 (Bérczi et al. 2005), however, revealed the importance of the R72 residue (the R in the middle of the predicted ASC-binding motive) in the ASC-dependent reduction kinetics but did not verify the importance of the appropriate Lys residue, K83. It should be noted that K85 in the bovine CGCyt-*b*561 refers to the K83 in the mouse CGCyt-*b*561 and K80 in the TCyt-*b*561 sequence (Fig. 2). Also, R72 in the mouse CGCyt-*b*561 refers to R74 in the bovine CGCyt-*b*561. However, there is no R residue in the vicinity of the ASC-binding motive (Fig. 2, boxed area) in the Arabidopsis TCyt-*b*561 but there are K residues in TCyt-*b*561 at places where R residues are present in bovine or mouse CGCyt-*b*561 in the vicinity of the ASC-binding motive (K70 and K149 in TCyt-*b*561 as compared to R72 and R152 in mouse CGCyt-*b*561; see Fig. 2). Since the high-affinity binding of ASC to CGCyt-*b*561 has been connected to the presence of an R residue (namely of the R72 in mouse CGCyt-*b*561; Bérczi et al. 2005), we were interested in if any of the 5 K residues in the vicinity of these two R residues were able to play a distinguished role in ASC-dependent reduction kinetics of TCyt-*b*561.

When all 5 K residues (namely K70, K76, K79, K80, and K149; see Fig. 2) were one-by-one replaced by A, a minor but significant decrease (about 40%) in the specific content of TCyt-*b*561 in the yeast microsomal membrane fraction was observed only with the K80A mutant (Fig. 3). This observation was fully complemented by Western blot analyses (Fig. 3). As it was mentioned above, the K80 residue in Arabidopsis TCyt-*b*561 corresponds to K85 in the bovine and K83 in the mouse CGCyt-*b*561 sequences. Different experiments have attributed different importance to this particular lysine residue in ASC binding or in ASC-dependent reduction kinetics of CGCyt-*b*561 (Tsubaki et al 2000; Bérczi et al. 2005). Furthermore, when the ASC-reducibility (in the presence of 25 mM ASC) to dithionate-reducibility (in the presence of 2.5 mM dithionite) – the so-called percentage ASC-reducibility – was compared, the lowest value was obtained with the K80A mutant (Fig. 3). These results obtained with the K mutants revealed that any K-to-A replacement in the vicinity of the ASC-binding motive in the CB domain could dramatically change neither the ASC-reducibility nor the expression of TCyt-*b*561.

In agreement with earlier kinetic studies on the recombinant mouse CGCyt-*b*561, the ASC-dependent reduction kinetics of all 5 K-to-A mutants could be explained only by assuming the presence of two "ASC-binding sites" with different affinity on the TCyt-*b*561:

$$[Cyt-b561]_{reduced} = \frac{A_1[ASC]}{K_1+[ASC]} + \frac{A_2[ASC]}{K_2+[ASC]},$$

where A_1 and A_2 are fitting parameters (referring to the reduction of either heme in TCyt-*b*561), K_1 and K_2 are the high-affinity and low-affinity binding constants, respectively. While K_1 seemed to be hardly influenced by any K-to-A replacement (data not shown), K_2 for the K80A mutant was significantly higher (about 10-fold) than that was for any other K-to-A mutant or for the non-mutated TCyt-*b*561 (Fig. 4). These results show that K80 in Arabidopsis TCyt-b561 plays a distinguished role in the ASC-dependent reduction kinetics of TCyt-b561.

In summary, H-to-A mutation of either His residues coordinating the high-potential heme in TCyt-*b*561 is lethal. H-to-A mutation at either His residues coordinating the low-potential heme in TCyt-*b*561 reduces significantly the expression level of the protein. Introduction of a His_6-tag at the C terminus of the TCyt-*b*561 decreases less the expression

level than that of a His_6-tag at the N terminus or a FLAG-tag at the C terminus. The ASC-dependent reduction of the recombinant Arabidopsis TCyt-*b*561 can be explained by assuming two "ASC-binding sites" with different affinity. It seems that K80 plays an important role in the ASC-dependent reduction of TCyt-*b*561.

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Substrate-dependent reduction of a recombinant chromaffin granule Cyt-*b*561 and its R72A mutant

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ABSTRACT Cytochrome *b*561 (Cyt-*b*561) proteins constitute a family of integral membrane proteins, catalyzing ASC-driven trans-membrane electron transport. Numerous isoforms of Cyt-*b*561 are present in invertebrates, vertebrates, and plants. The only protein of this family, however, which has been characterized in details at both biophysical, biochemical and physiological levels so far, is the bovine chromaffin granule Cyt-*b*561 (CGCyt-*b*561). Recently, both the bovine and the mouse CGCyt-*b*561 has been expressed in yeast cells and the recombinant proteins were shown to have biophysical properties similar to the native bovine CGCyt-*b*561. We have expressed the mouse CGCyt-*b*561 with a His_6-tag at the C terminus (CGCyt-*b*561(C6H)) in yeast (*Saccharomyces serevisiae*) cells and studied the reduction of CGCyt-*b*561(C6H) in the presence of different natural reducing agents. Besides the well-known natural reductant ascorbate (ASC) and the often-used artificial reductant dithionate, NADH, GSH, and dihydrolipoic acid (DHLA), also reduced the fully oxidized protein. Interestingly however, NADPH was not effective at all. When the same reductants were tested with the R72A mutant of CGCyt-*b*561(C6H), a mutant with impaired ASC-dependent reducibility, neither pyridine-dinucleotides could reduce the R72A mutant. DHLA-dependent and ASC-dependent reduction kinetics were very similar in case of the R72A mutant but differed in case of CGCyt-*b*561. These results raise the question of how many natural reductants the CGCyt-*b*561 may utilize *in vivo*.

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KEY WORDS

ASC reduction
arginine residue
dihydrolipoic acid
His_6-tagged protein
cytochrome b561
reductants

Cytochromes *b*561 (Cyts-*b*561) constitute a newly described family of membrane proteins, present in both plants and animals (Asard et al. 2001; Tsubaki et al. 2005). Cyts-*b*561 contain two *b*-type heme molecules per molecule of protein (Tsubaki et al. 1997). There are 6 trans-membrane (TM) α-helices in Cyts-*b*561 and TM2 through TM5 define the so-called CB domain (Pointing 2001). One His residue on each α-helix of the CB domain is highly conserved and participates in coordination of the two hemes (Okuyama et al. 1998). Structure prediction studies indicate that, CGCyt-*b*561 follows the "Four-helix motif" (Lancaster 2002), where the extravesicular heme is coordinated by His residues on TM3 and TM5, and the intra vesicular heme is coordinated by His residues on TM2 and TM4 (Bashtovyy 20003).

Cyt-*b*561 of the chromaffin granules (CGCyt-*b*561) is the best-characterized member of the Cyt-*b*561 protein family. CGCyt-*b*561 is able to mediate trans-membrane electron transfer from ASC present on the extra-vesicular side to monodehydroascorbate (MDHA) present on the intra-vesicular side of the protein (Kent and Fleming 1987; Fleming and Kent 1991). Two regions of CGCyt-*b*561 have been identified as putative substrate binding sites, the partially conserved region ^{69}ALLVYRVFR^{74} (numbering is for the bovine CG-Cyt-*b*561) present at the end of TM2 helps in ASC binding while the well-conserved motif of ^{120}SLHSW^{125}, present on the intra-vesicular side of CGCyt-*b*561 presumably aids in binding of MDHA. Chemical modification studies on bovine CGCyt-*b*561, by treating the protein with diethyl-pyrocarbonate (DEPC) showed the importance of His residues in mediating trans-membrane electron transfer. DEPC has the ability to interact with His, Lys and Tyr residues and covalently modify them. CGCyt-*b*561, treated with DEPC, had altered properties with respect to substrate binding. Mass spectrometry revealed that H88, H159, and K85 in the bovine CGCyt-*b*561 were modified. To His residues were assigned a role in the coordination of extra-vesicular heme and K85 was thought to be involved in ASC binding (Tsubaki et al. 2000). Recent studies with recombinant mouse CGCyt-*b*561 revealed the importance of an Arg residue (R72 in the mouse CGCyt-*b*561 sequence) in ASC-dependent reduction of the protein as well as identified the essential His residues coordinating the two hemes and responsible for the optical properties of CGCyt-*b*561 (Bérczi et al. 2005).

In the near past, new members of the Cyt-*b*561 protein family were identified. One such protein is the so-called duodenal Cyt-*b*561 (DCyt-*b*561) was identified by subtractive cloning strategy (McKie et al. 2001). DCyt-b561 was shown to localize at the brush border membranes of the duodenum

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and exhibited ferric reductase activity when transiently expressed in Xenopus oocytes and in intestinal cell lines (McKie et al. 2001). Other new member of the Cyt-*b*561 protein family includes the lysosomal Cyt-*b*561 (LCyt-*b*561), named after its localization in lysosomes of macrophages and sertoli cells. LCyt-*b*561 is similar to CGCyt-*b*561 in spectral characteristics and is possibly involved in iron metabolism (Deliang Zhang and Han Asard, personal communication). A fourth isoform, tentatively named "tumor suppressor Cyt-*b*561" (gene name:101F6, herein abbreviated as TSCyt-*b*561) was identified in the 120kb tumor homozygous deletion region of chromosome 21 found in breast and lung cancers (Ji et al. 2002). Over-expression of TSCyt-*b*561 inhibited tumor cell growth by induction of apoptosis and altering cell cycle progression. The biochemical characterization and understanding the localization and physiological function of these newly identified Cyts-*b*561 are awaited.

Based on primary sequence conservation and predicted structural features, four putative Cyt-*b*561 genes have been identified in *Arabidopsis thaliana* (Asard et al. 2001). Although there is limited homology between the predicted plant and animal sequences (approximately 30%), the presence of certain conserved residues and structural features support the similarity between the functions of these proteins. One of plant Cyts-*b*561 has already been over-expressed in yeast (*Saccharomyces serevisiae*) cells and the recombinant protein has been characterized to some extent (Griesen et al. 2004). The protein seems to be localized at the plant tonoplast (TCyt-*b*561) and encodes for an ASC-reducible Cyt-*b*561 with absorbance characteristics similar to that of CGCyts-*b*561. Presence of a Cyt-*b*561 in plant tonoplast was also obtained by characterizing an ASC-reducible *b*-type cytochrome in tonoplast-enriched bean membrane fraction (Preger et al. 2005).

Hardly any is known about the substrate specificity of Cyts-*b*561. The only study in this respect was done by Terland and Flatmark (1980) on highly-purified chromaffin granule ghosts from bovine adrenal medulla. It was shown that less than 1% of CGCyt-*b*561 was reduced by 0.1 mM NADH at pH 6.5 in 2 min. It is shown in this paper that the highly-purified recombinant mouse CGCyt-*b*561 can be reduced by millimolar concentrations of natural reductants, and dihydrolipoic acid (DHLA) seems to be as effective reductant as ASC.

Table 1. Effect of reductants on purified recombinant mouse CGCyt-*b*561 and its R72A mutant. For reference values, reduction at 25 mM of ASC is given as 100%; the 100% refers to 2.45 ± 0.75 nmol mg^{-1} and 1.20 ± 0.45 nmol mg^{-1} for CGCyt-*b*561 and its R72A mutant, respectively. All other measurements were at 2.5 mM of reducing agents. Values are means ± deviation from the means of two independent experiments.

Reducing agent potential	Percentage reduction		Midpoint redox
	CGCyt-b561	R72A-CGCyt-b561	$E_{m,7}$ (mV)
ASC (25 mM, ref.)	100	100	+320
ASC	75 ± 6	71 ± 1	+320 [b]
GSH	18 [a]	11 [a]	–230 [c]
DHLA	75 ± 5	69 [a]	–320 [d]
NADH	24 ± 5	0	–330 [c]
NADPH	0	0	–330 [c]
DTH	158 ± 6	122.2 ± 1	–480 [e]

[a] measured only once
[b] Washko et al. (1992), [c] Foyer and Noctor (2005), [d] Moini et al. (2002), [e] Mayhew (1978),

Materials and Methods

Molecular biology works

Yeast expression vector pESC-His containing the mouse CGCyt-*b*561 gene (Bérczi et al. 2005) was used as a template for PCR reactions to generate N and C-terminal His-tagged proteins. Primers were designed as given by Liu et al. (2005). Standard PCR methods were used to amplify the gene and amplified sequences were confirmed by DNA-sequencing at the University of Nebraska – Lincoln Genomic Core Research Facility.

For transformation, yeast cells (*Saccharomyces cerevisiae*, strain YPH499, ura3-52 lys-801^{amber} ade2-101^{ochre} trp1-Δ63 his3Δ200 leu2-Δ1) were grown in synthetic dextrose minimal medium (YPAD, Stratagene, La Jolla, CA) and transformation was performed according to manufactures instructions. Transformed lines were selected on SD dropout medium, lacking His (SD-His). For the induction of protein expression, overnight cultures were grown in SD-His, and transferred to 450 ml of synthetic galactose minimal medium (SG-His) containing 2% (w/v) galactose.

Yeast membrane preparation and stripping

Cells grown as 4 x 450 ml cultures were collected by low-speed centrifugation (5,000 g_{max} and room temperature for 10 min) when the OD_{600} reached a value of 0.8 to 1.0. Collected

Table 2. Kinetic constants obtained from data analysis of concentration dependent reduction by ASC and DHLA of CGCyt-*b*561 (Wild) and its R72A mutant (Fig. 1). Data analysis was performed with Origin5.0 software. Values are for the best fit to the experimental points with their uncertainty provided by Origin5.0.

CGCyt-b561	Reductant	"Apparent binding constant" for the High-affinity site (mM)	Low-affinity site (mM)
Wild	ASC	0.021 ± 0.006	1.50 ± 0.50
	DHLA	0.071 ± 0.020	-
R72A	ASC	-	1.48 ± 0.14
	DHLA	-	1.56 ± 0.10

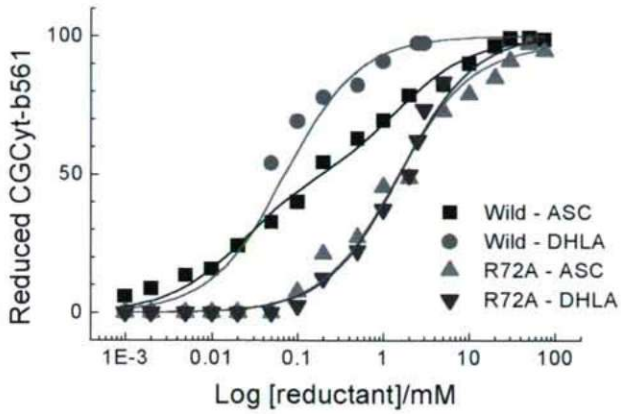

Figure 1. Concentration-dependent reduction by ASC or DHLA of highly-purified recombinant CGCyt-*b*561 (Wild) and its R72A mutant (R72A). For comparison, results are shown as percentage reduction of proteins. Experimental results are means from two independent experiments. Continuous lines are theoretical curves fitted by Origin5.0 software.

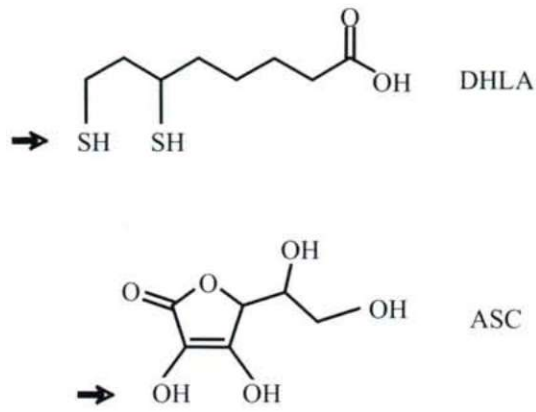

Figure 2. Chemical structure for ascorbic acid (ASC) and dihydrolipoic acid (DHLA) in their undissiociated forms. Arrows indicate the –SH and –OH groups discussed in the text.

cells were washed once with ice-cold homogenization buffer (50 mM MOPS-KOH, pH 7.0, 5 mM EDTA, 100 mM KCl, 100 mM sucrose) and pelleted as above. Microsomal membrane preparation and stripping of membranes were as described recently by Bérczi et al. (2005). Stripped membrane fractions were re-suspended in storage buffer (20 mM MES-Tris, pH 6.8, containing 2% (w/v) glycerol or 50 mM $NaH_2PO_4.H_2O$, pH 6.8, 2% (w/v) glycerol), and stored at –80°C until use.

Protein solubilization and purification by affinity chromatography

Membrane vesicles (about 80 mg protein) were resuspended in 50 mM $NaH_2PO_4.H_2O$, pH 6.8, 2% (w/v) glycerol, 1% (w/v) sucrose monolaurate (SML) at 2 mg ml^{-1} protein concentration. The mixture was incubated on a rocker at room temperature for a period of 60-90 min. Insoluble material was pelleted by high-speed centrifugation (Beckman Avanti centrifuge, JA-25.50 rotor) at 75,000 g_{max} and 4°C for 90 min. The supernatant containing the detergent-solubilized proteins was supplemented with 500 mM NaCl and 10 mM imidazole, the pH was adjusted to 7.8 and then mixed with 1 ml (bed volume) of Ni-NTA agarose resin (Ni-NTA His-bind Superflow, Novagen, Madison, WI) that had been pre-equilibrated with 50 mM $NaH_2PO_4.H_2O$, pH 7.8, containing 10% (w/v) glycerol, 1%(w/v) SML, 500 mM NaCl and 10 mM imidazole. The agarose resin was incubated with the solubilized proteins at room for 60 min. After incubation, the Ni-NTA agarose resin with bound proteins was collected in a 5-ml disposable column and washed thrice with 5 bed volumes of 50 mM $NaH_2PO_4.H_2O$, pH 7.8, containing 10% (w/v) glycerol, 0.1% (w/v) SML, 500 mM NaCl and 10 mM imidazole. His-tagged proteins were eluted with 6 bed volumes of 50 mM $NaH_2PO_4.H_2O$, pH 7.0, containing 10% (w/v) glycerol, 0.1%(w/v) SML, 150 mM NaCl and 250 mM imidazole. The eluate was concentrated by centrifugation using Centricon-YM100 centrifugal filter unit (Millipore, Bedford, MA) and desalted by using a PD-10 desalting column (Amersham Biosciences, Uppsala, Sweden). The desalted fraction was concentrated again and stored at –80°C until use.

Absorption spectroscopy

Absorption spectra were recorded in split beam mode (with buffer as reference) with an OLIS-updated SLM-Aminco DW2000 spectrophotometer (OLIS Co., Bogart, GA) with 1 nm slit-width, 0.5 nm s^{-1} scan rate, and under continuous stirring. First, Cyts were oxidized by addition of ferricyanide (0.5 mM, FeCN) and the fully oxidized spectra were recorded. Then, ASC-reduced (25 mM ASC) and ASC +dithionite-reduced (after addition of Na-dithionite crystals) spectra were obtained at room temperature. When improvement of the signal to noise ratio was needed, multiple scans were averaged. Cyt *b* amounts were calculated from the reduced-minus-oxidized difference spectra, obtained by subtracting the FeCN oxidized spectra from the ASC- or dithionite-reduced spectra, using a millimolar extinction coefficient of 30 mM^{-1} cm^{-1} (Tsubaki et al. 1997; Liu et al. 2005).

Results and Discussion

Although the major property of members of the Cyt-*b*561 protein family is their ASC-reducibility, hardly any work is known for studying the effect of other native and putative reducing agents present together with ASC in cells. Reason for this might be rooting in the facts that no pyridine nucleotide-binding site can be identified in any Cyt-*b*561 sequences and midpoint redox potentials for the most common reductants

in the cytosol are hundreds of millivolts lower than that for Cyts-*b*561.

Earlier studies on intact chromaffin secretory vesicles indicated that intra-granular ASC is regenerated by extra-granular ASC, however, other reducing agents, such as GSH, NADH and NADPH did not support this process (Dhariwal et al. 1991). Also, the ability of serotonin-containing dense granules of platelets, to accept electrons from NADH and NADHP were studied. Since serotonin-containing granule resembles that of chromaffin granules, the reduction was compared to CGCyt-*b*561 (Johnson and Scarpa 1981). In this study it was found that NADH could donate electrons to CGCyt-*b*561, but at a slower rate when compared to ASC. NADPH was unable to supply electrons. The ability of such physiologically relevant reducing agents to donate electrons to purified CGCyt-*b*561 has not yet been documented. Using the purified recombinant CGCyt-*b*561, the ability of NADH, NADPH, GSH, DHLA and dithionite (DTH) to reduce the CGCyt-*b*561 was tested.

Table 1 summarizes our results obtained when reduction of both FeCN-oxidixed recombinant mouse CGCyt-*b*561 and its R72A mutants were studied in the presence of 2.5 mM of both native and artificial reducing agents. It is clear that GSH as well as pyridine nucleotides are bad reducing agents for CGCyt-*b*561 but dithiol-containing reagents are capable of reducing both CGCyt-*b*561 and its R72A mutant. The physiological amounts of GSH in the cells vary from 0.5 mM to 10 mM (Wu et al. 2004) and that of NADH and NADPH vary from μM to mM range (Kirsch and de Groot 2001). Therefore the relevance of the observed reductions of CGCyt-*b*561 should be interpreted cautiously, as the values for NAD(P)H used (2.5 mM) are relatively high when compared to physiological levels. DHLA is present in mM concentrations in cells and is synthesized both in plants and animals (Packer et al. 1995; Moini et al. 2002). It is particularly interesting that (1) DHLA is as effective as ASC and (2) reduction by NADH is lost when R72 is replaced by A. This latter observation adds some emphasis to the predicted importance of R72 in the reduction mechanism of CGCyt-*b*561. There is not correlation between the reducing efficacy and midpoint redox potential of reductants tested.

It has been shown recently (Bérczi et al. 2005) that R72 seems to be responsible for the high-affinity ASC-binding of CGCyt-*b*561. It was concluded from (a) the lack of ASC-reducibility of R72A-CGCyt-b561 below 0.1 mM of [ASC] and (b) the kinetic parameters of data analysis. Concentration-dependent reduction of R72A-CGCyt-*b*561 by DHLA was very similar to that by ASC (Fig. 1). DHLA seems to have no low-affinity binding to CGCyt-*b*561. Data analysis of experiments (Table 2) revealed that reduction by ASC and DHLA might follow similar kinetics and reduction of R72A-CGCyt-*b*561 occurs only via low-affinity bindings of reductants. It is believed that reducing activity of DHLA resides in its –SH groups. Comparing (a) the chemical structure of ASC and DHLA (Fig. 2), (b) the midpoint redox potential values of ASC and DHLA (Table 1), and observing (c) the very similar reduction kinetics with these two reductants and the R72A mutant (Fig. 1), and (d) the slight difference between the high-affinity binding constants for ASC and DHLA, it might be probable that redox reactions at low reductant concentrations occur via specific interactions between the reductants and CGCyt-*b*561; for instance, R72 interacts with the two –SH groups on DHLA in a similar way as it does with the two –OH groups on ASC. Further studies are needed to clarifying the reduction mechanism by either ASC or DHLA.

In summary, it is shown that affinity-purified recombinant mouse CGCyt-*b*561 and its R72 mutant can be reduced not only by ASC (and DTH) but also by other native reductants. The reducing mechanism by DHLA shows similar properties to that by ASC. In both cases, an Arg residue in the predicted ASC-binding motive (R72) seems to play a key role in the high-affinity reduction of CGCyt-*b*561.

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Role of membrane redox in aging-related diseases

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ABSTRACT A number of different ECTO-NOX forms have been described as being connected with aging-related diseases. The constitutive form, CNOX, serves as a terminal oxidase of plasma membrane electron transport and functions in the growth process. tNOX is present in addition to CNOX on the surface of all cancer cells and contributes to the unregulated growth characteristic of cancer cells. An age-related ECTO-NOX, arNOX, generates superoxide and may contribute to age-related generation of reactive oxygen species. ECTO-NOX proteins and prions share properties in common as do amyloid-forming proteins of various neurodegenerative disorders. A better understanding of ECTO-NOX proteins may lead to new therapeutic strategies for these several age-related disorders. **Acta Biol Szeged 50(1-2):67-69 (2006)**

KEY WORDS

ECTO-NOX proteins
aging
cancer
Alzheimer's disease
neurodegenerative diseases

The most thoroughly studied involvement of an ECTO-NOX protein in disease has been that of tNOX and cancer (Morré 1998; Cho et al. 2002). More recently tNOX has been shown to exhibit properties of a prion and various amyloid generating proteins of neurodegenerative diseases (Alzheimer's β-amyloid, Parkinson's α-synuclein). The mouse prion has properties in common with NOX proteins (Morré and Morré 2003; Kim and Morré 2004; Markert et al. 2004). We also have described an aging-related ECTO-NOX potentially involved in the generation of reactive oxygen species at the cell surface.

Methods

The oxidative activity of the ECTO-NOX proteins is most often measured from the decrease in A_{340} from the oxidation of NADH (or NADPH). With turbid membrane preparations, use of a dual beam instrument with the photo multiplier tube proximal to the sample, is essential to reduce light scattering error in continuous measurements. The Aminco SLM 2000 is a double beam, dual wavelength grating instrument designed for these types of measurements.

Results and Discussion

tNOX and cancer

The cancer-associated, drug-responsive and constitutively-activated ECTO-NOX form (designated tNOX) is present at the cell surface of invasive human cancers (Morré 1998; Cho et al. 2002; Chueh et al. 2004). tNOX is shed into the circulation and, together with the cell surface form, provides a potential diagnostic drug and vaccine target (Cho et al. 2002). The normal constitutive NOX (CNOX) form is hormone- and growth factor-regulated and responds to agents that control growth and development (Bruno et al. 1992). When a cell divides, it must reach some certain minimal size to divide again. When one or more NOX proteins are inhibited, cell enlargement is slowed or blocked. The resultant small cells fail to divide and ultimately undergo programmed cell death (apoptosis; Morré and Morré 2003).

The ECTO-NOX form associated with human cancers, tNOX, differs from the constitutive form of normal cells (CNOX) primarily in that its activities are blocked by quinone-site inhibitors many of which have anticancer activity (Morré 1998). Examples include adriamycin, the antitumor sulfonylureas, the antitumor vanilloids (capsaicin), the principal tea catechin, (-)-epigallocatechin-3-gallate, and several known differentiating agents including antitumor retinoids, sodium phenylacetate and calcitriol. These agents are largely without effect on the CNOX activity of normal cells. They inhibit both tNOX and growth of cancer cells at potentially therapeutic dosage levels without inhibiting CNOX or growth of non-cancer cells. Drug inhibition by tNOX has served as a defining tNOX characteristic used to guide its isolation and molecular characterization. The drug responsive tNOX appears to arise as a splice variant from a single tNOX gene different from that encoding CNOX and is delivered to the cell surface in a processed form.

NOX proteins are released from cells into the circulation. Sera of cancer patients contain both tNOX and CNOX proteins (Morré et al. 1997; Morré and Reust 1997; Cho et al. 2002). Sera of healthy volunteers or of patients with diseases other than cancer contain only the CNOX form. tNOX has been found in sera of patients with all major forms of cancer including leukemia and lymphomas (Morré et al. 1997; Morré and Reust 1997; Cho et al. 2002) and serves as the basis for a cancer diagnostic protocol under development.

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Table 1. Amyloid-forming proteins that exhibit periodic (copper-dependent) oscillations in NADH oxidation.

CNOX (CLOX)[1]	24 min	
tNOX[2]	22 min	ECTO-NOX proteins
arNOX[3]	26 min	
Aβ 1-42	24 min	(Alzheimer's disease)
Mouse prion	24 min	(Spongiform encephalopathies)
α-Synuclein	54 min	(Parkinson's disease)

[1]Constitutive NOX (normal cells and tissues)
[2]Tumor NOX (cancer specific)
[3]Age-related NOX (aged > 70 y individuals)

ECTO-NOX proteins have characteristics of prions

ECTO-NOX proteins and prions share similar properties (Kelker et al. 2001). The ability to form insoluble aggregates and the presence of bound copper are ECTO-NOX protein characteristics (Morré and Morré 2003). Other unusual characteristics exhibited by tNOX and shown with other ECTO-NOX proteins include resistance to proteases, resistance to cyanogen bromide fragmentation, and an ability to form amyloid filaments closely resembling those of transmissible spongiform encephalopathies. Additionally, tNOX imparts protease resistance to a normally protease-susceptible protein as is characteristic of PrP^{sc} (PrP^{res}), the presumed infective and proteinase K-resistant scrapie prion form. Recombinant mouse prion exhibited a copper dependent pattern of oscillating and alternating NADH oxidase and disulfide-thiol interchange with a 24 min period indistinguishable from that of CNOX (Kim and Morré 2004).

Aging

An aging-related ECTO-NOX protein (arNOX) of human sera and buffy coat fractions of individuals > 60 y with a potential role in atherogenesis generates superoxide (Morré et al. 2000; 2003). Superoxide production capable of oxidizing circulating lipoproteins and other extracellular targets are measured by the reduction of ferricytochrome c. Activity is inhibited by both superoxide dismutase (SOD) and coenzyme Q_{10}. Coenzyme Q_0, Q_2 and Q_6 do not inhibit. The activity is reduced or absent from sera and/or buffy coat fractions of younger individuals (20 – 40 y) but appears to be widely distributed among other aged systems including late-passage cultured cells and senescing plant organs.

The superoxide generated is also active in the reduction of tetrazolium salts such as XTT (Na 3'-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid) leading to colored formazan formation. Other NOX proteins lack this activity.

arNOX proteins (Morré 1998; Morré et al. 1999) and other ECTO-NOX proteins (de Grey 1999) have been postulated to link the accumulation of lesions in mitochondrial DNA to cell surface accumulations of reactive oxygen species as one consequence of their role as a terminal oxidase in a plasma membrane electron transport chain. Cells with functionally deficient mitochondria become characterized by an anaerobic metabolism. NADH accumulated from the glycolytic production of ATP and an elevated plasma membrane electron transport activity are then necessary to maintain the NAD^+/NADH homeostasis essential for survival.

Neurodegenerative disorders

tNOX aggregates in the form of enzymatically inactive amyloid rods and open cylinders (rings) were observed by high resolution electron microscopy (Kelker et al. 2001). These structures were virtually indistinguishable from corresponding aggregates associated with neurodegenerative amyloid-forming proteins.

Observations were extended to Alzheimer's Aβ 1-43 peptide (Markert et al. 2004) and α-synuclein of Parkinson's disease (Morré and Morré 2003). The Alzheimer's Aβ peptide exhibits copper-dependent oscillations in NADH oxidase activity similar to that of tNOX and the mouse prion also with a 24 min period length (Morré and Morré 2003). The Aβ peptide both lacks a cysteine and is unable to carry out disulfide-thiol interchange. α-Synuclein also has a copper-dependent and oscillating NADH oxidase activity but the period length is 54 min. The α-synuclein has not been evaluated for disulfide-thiol interchange activity. These various relationships are summarized in Table 1. Complete amino acid sequences are known for each of the proteins listed in Table 1. There is virtually no sequence similarity. Even though all bind copper, the copper binding strategies differ. For tNOX, the putative copper site is a HVH and a 3rd H downstream conserved with superoxide dismutase. The mouse prion utilizes the hexa repeats. For α-synuclein and Alzheimer's Aβ, a combination of tyrosines are utilized. It is unlikely that CNOX will exhibit substantial sequence similarity to tNOX based on data base searches and antisera specificity. Yet all examples in Table 1 bind adenine nucleotide (NADH) although the amino acid sequences within the putative binding regions differ.

Possibly the best source of a new set of tools to understand ECTO-NOX periodicity and oxidative function is now provided by the Alzheimer's Aβ peptide of 43 amino acids in length. The copper site is known and a putative NADH site is found near the C-terminus as in tNOX in the vicinity of M-35. We are hopeful that a detailed analysis of various modifications within the Aβ peptide will begin to shed light on how ECTO-NOX proteins keep time and carry out their oxidative functions.

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A CNOX-like protein disulfide-thiol interchange activity of the cell surface of mouse sperm

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ABSTRACT Intact frozen mouse sperm were analyzed for the presence of ECTO-NOX-like protein disulfide-thiol interchange activity. Activity was determined both from the cleavage of a dithiodipyridine substrate and from the restoration of activity to scrambled and inactive ribonuclease. An activity was found using both methods of activity determination. The activity was resistant to inhibition by both capsaicin and bacitracin. The activity, which oscillated in the characteristic manner of ECTO-NOX proteins, was characterized by a pattern of five maxima and an overall period length of 24 min. Three of the five maxima were separated by an interval of 6 min and the remaining maxima were separated by intervals of 4.5 min to generate the repeating pattern with a period length of 24 min. The activity pattern was unusual in that all five of the maxima within the 24 min repeat were of approximately equal specific activity. Normally, for somatic cells, the first two maxima are involved in NADH oxidation and less pronounced in terms of protein disulfide-thiol interchange than are the remaining three.
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KEY WORDS

mouse sperm
protein disulfide isomerase
ECTO-NOX
NADH oxidase

There have been reports of protein disulfide isomerase associated with plasma membranes of several cell types (Kroning et al. 1994) and multiple functions have been attributed to the protein (Zair et al. 1999). Protein disulfide isomerase, for example, assists protein folding by expressing both an isomerase and a chaperone-like activity (Wang 1998; Chen et al. 1999). A protein disulfide isomerase-like activity has been found associated with the plasma membranes of sperm (Bohring et al. 2001) and has been suggested to be important for capacitation and the acrosome reaction. A similar activity has been associated with the acrosome (Ohtani et al. 1993).

In the present report a protein disulfide isomerase-like activity (protein disulfide thiol interchange) is demonstrated for the cell surface of mouse sperm based on assays using cell impermeant substrates. The activity has characteristics of a constitutive ECTO-NOX (CNOX) activity as is associated with plasma membranes of a variety of cells and tissues (Morré 1998) rather than that of a classical protein disulfide isomerase (e.g., ER 60) typically associated with the endoplasmic reticulum (Essex et al. 2001).

Methods

Frozen mouse sperm was supplied by Dr. Diego A. Ellerman, University of California, Davis, California (Ellerman et al. 2003).

Oxidation of NADH was determined from the disappearance of NADH measured at 340 nm in a reaction mixture containing 25 mM Tris-MES (pH 7.2), 100 μM GSH, 1 mM KCN to inhibit mitochondrial oxidase activity, and 150 μM NADH at 37°C with temperature control (± 0.5°C) and stirring (Morré and Morré 2003a, 2003b). Activities were measured using paired Hitachi U3210 spectrophotometers with continuous recording. Assays were initiated by addition of NADH. Assays were for 1 min and were repeated on the same sample every 1.5 min for the times indicated. A millimolar extinction coefficient of 6.22 was used to determine specific activity.

Protein disulfide-thiol interchange was determined spectrophotometrically from the activation of scrambled and inactive RNase with cleavage of cCMP measured spectrophotometrically as the assay (Lyles and Gilbert 1991) or from the cleavage of an artificial dithiodipyridine substrate (Morré et al. 1999).

Proteins were estimated by the bichinchoninic acid method (Smith et al. 1985). Bovine serum albumin was the standard.

Results

Mouse sperm exhibited a classical protein disulfide-thiol interchange (protein disulfide isomerase) activity as determined by the ability to activate scrambled and inactive ribonuclease (Fig. 1). cCMP was used as the substrate in a standard spectrophotometric assay (Lyles and Gilbert 1991). In contrast to a classical protein disulfide isomerase, the activation was not linear but periodic. Periods of activation were separated by periods of lesser activity at intervals of ca 24 min (Fig. 1, upper curve, arrows). In the absence of sperm, the scrambled ribonuclease remained inactive (Fig. 1, lower curve).

Assay of protein disulfide-thiol interchange using a dithiodipyridine (DTDP) substrate also revealed an oscil-

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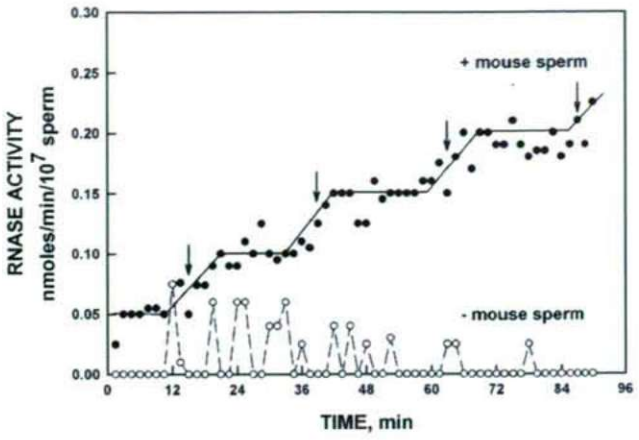

Figure 1. Protein disulfide-thiol interchange activity of mouse sperm measured from the activation of scrambled RNase. Preparations plus (upper curve) or minus (lower curve) sperm were assayed in parallel. The ribonuclease assay measured spectrophotometrically as the cleavage of cCMP (Lyles and Gilbert 1991).

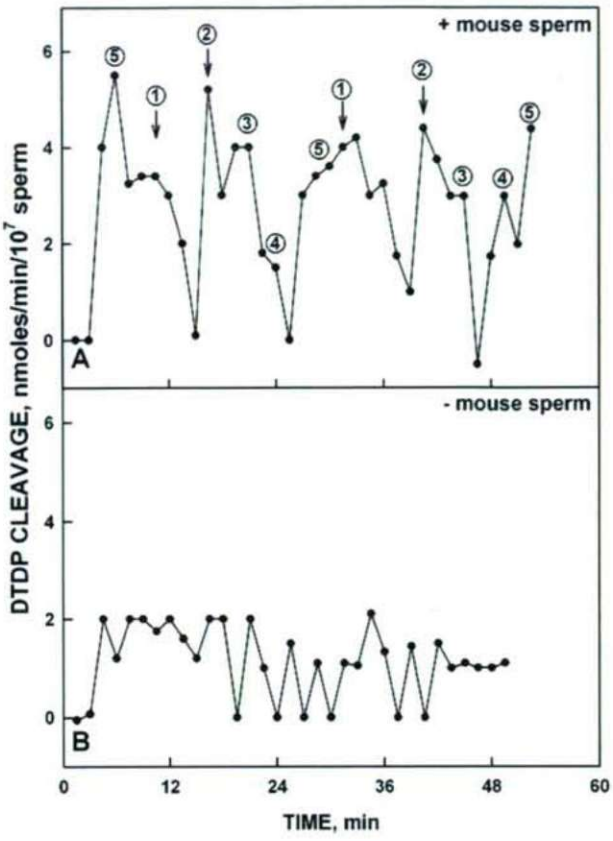

Figure 2. Rate of protein disulfide-thiol interchange of mouse sperm as determined by cleavage of dithiodipyridine (DTDP; Morré et al. 1999). Preparation plus (B) or minus (A) sperm were assayed in parallel.

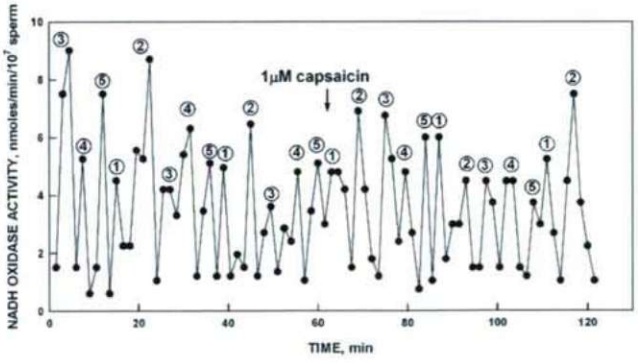

Figure 3. Rate of NADH oxidation by mouse sperm. The activity exhibited the typical CNOX activity pattern of oscillations with two maxima separated by intervals of 6 min and the remaining maxima separated by intervals of 4.5 min to generate a 24 min period (6 + 4 x 4.5). Capsaicin (1 μM) was added after 60 min.

lating pattern of activity also dependent upon the presence of sperm (Fig. 2B). Activity in the absence of sperm did not oscillate (Fig. 2A).

Since both the scrambled ribonuclease and the DTDP substrates revealed oscillatory patterns, the sperm were next assayed for the ability to oxidize NADH (Fig. 3). Again an oscillating pattern was observed consisting of five maxima, two of which ① and ② were separated by six min and three additional maxima ③, ④ and ⑤ were separated from each other and from the ① and ② doublet by intervals of 4.5 min to generate a period length of 24 min (6 + 4 X 4.5 min). Capsaicin added after 60 min was without effect on the activity as was bacitracin (not shown).

Parallel measurements of NADH oxidation (Fig. 4A) and DTDP cleavage (Fig. 4B) showed that both activities followed the same oscillatory pattern. These measurements were made simultaneously using two aliquots of the same sperm sample but with the two different substrates using paired spectrophotometers. With NADH oxidation, as in Figure 3, the 5 maxima of the 24 min repeating set of oscillations were of nearly equal magnitude (Fig. 4A). However, with the DTDP substrate to measure protein disulfide-thiol interchange (Fig. 4B), on average maxima labeled ③, ④ and ⑤ were somewhat greater than those labeled ① and ②.

Discussion

Mouse sperm exhibited a protein disulfide isomerase-like activity with characteristics more typical of that of constitutive ECTO-NOX proteins than that of a classical protein disulfide isomerase (Morré and Morré 2003a, 2003b). As is characteristic of ECTO-NOX proteins in general, their enzymatic activities oscillate in a characteristic pattern that imparts a time keeping attribute to the protein (Morré et al. 2002). ECTO-NOX proteins are located on the external cell surface, loosely bound, and exhibit both NADH (or

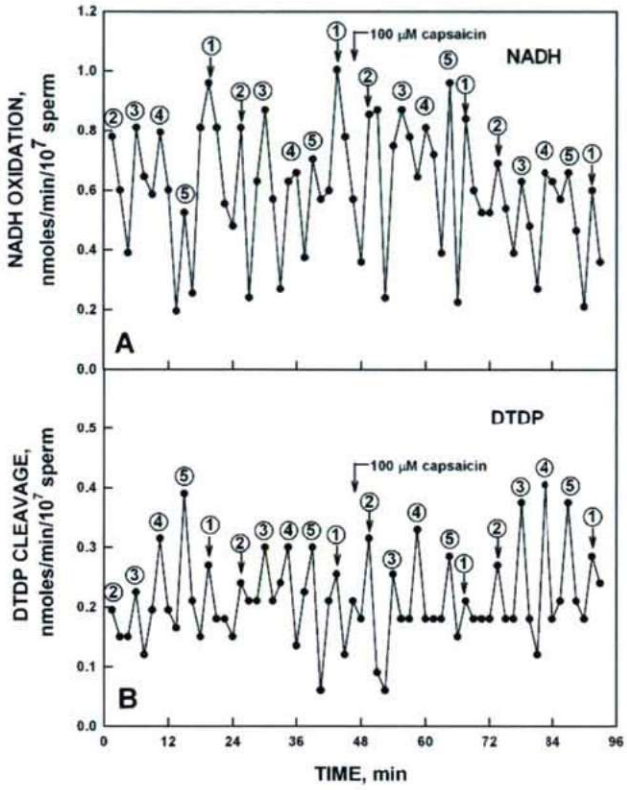

Figure 4. Rate of NADH oxidation (A) and rate of protein disulfide-thiol interchange (DTDP assay) (B) measured in parallel. Capsaicin (100 μM) was added after 45 min of incubation.

hydroquinone) oxidase activity and protein disulfide-thiol interchange. Normally the two activities alternate with two maxima separated by six min favoring NADH oxidation and the three maximum separated by 4.5 min favoring protein disulfide-thiol interchange.

The activity observed with the mouse sperm is unusual in that there is no distinction in activity between any of the maxima for NADH oxidation. They are all approximately equal. However, with activation of RNAse, periods of activity alternate with periods of inactivity as observed previously for both CNOX (Results unpublished) and a cancer-specific ECTO-NOX designated tNOX (Chueh et al. 2002). Similar results were observed in the experiments of Figure 4, where protein disulfide thiol interchange was augmented during the portion of the cycle attributed to maxima labeled ③, ④ and ⑤. These observations have mechanistic implications in that during this part of the cycle, the protein appears to have the capability, previously unobserved, of simultaneous oxidation of NADH and protein disulfide-thiol interchange.

tNOX differs from CNOX by being responsive to quinone site inhibitors such as capsaicin (Morré et al. 1995). Since the sperm activity was unaffected by capsaicin, it is an activity other than tNOX. Resistance to bacitracin distinguishes it from the more classical protein disulfide isomerases (Essex et al. 2001). The characteristic C-X-X-C motif common to most, if not all, members of the protein disulfide isomerase family of proteins (Ohnishi et al. 1995) is missing from tNOX. Neither tNOX nor CNOX appear to contain bound flavin nor are their activities dependent upon addition of flavins (FAD or FMN). The protein disulfide thiol interchange catalyzed by mouse sperm most clearly fit the category of a non-flavin-containing ECTO-NOX with functions in time keeping and physical membrane displacement rather than having the traditional chaperone function attributed to the protein disulfide isomerases of the endoplasmic reticulum, for example.

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Membrane redox as an essential component of how cells increase in size following cell division

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ABSTRACT Under investigation is the hypothesis that cell enlargement in both plants and animals is not a passive process but the result of an ECTO-NOX-driven physical membrane displacement. Cell enlargement correlates with ECTO-NOX activity and is stimulated when ECTO-NOX activities are stimulated and inhibited when ECTO-NOX activities are inhibited. Both are blocked by thiol reagents. Additionally, cell enlargement emerges as having an energy requirement. An energy requirement is universal among membrane displacement models and is met at the cell surface through coupling with a plasma membrane-associated AAA-ATPase.
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KEY WORDS

2,4-D
cell enlargement
ECTO- NOX
growth
membrane-displacement

Cell enlargement is a necessary requisite for sustained growth of both plant (Taiz 1984) and animal (Baserga 1985) cells. However, a prevailing view for plants established early (Lockhart 1965), is that cell enlargement is the result of a passive yielding of cell walls in response to turgor pressure. In this report we combine information from various sources and cell free systems (Morré 1998b) together with more recent findings to suggest that cell enlargement in both plants and animals is the result of an active, energy-driven process mediated by ECTO-NOX (cell surface NADH or hydroquinone oxidases with protein disulfide-thiol interchange activity) proteins as a major physiological function.

Materials and Methods

NADH oxidase activity was determined as the disappearance of NADH measured at 340 nm in a reaction mixture containing 25 mM Tris-Mes buffer (pH 7.2), 1 mM KCN to inhibit any low levels of mitochondrial oxidase activity, and 150 μM NADH at 37°C with stirring. Activity was measured using a Hitachi U 3210 spectrophotometer as recorded over two intervals of 5 min each in the presence or absence of hormone or growth factor. A millimolar extinction coefficient of 6.22 was used to determine specific activity.

Other methods have been published in detail elsewhere and will not be repeated here (Eisinger and Morré 1968; Morré 1994, 1995, 1998a,b).

Results

Turgor pressure is not the driving force of cell enlargement in plants.

In early experiments, plant stem sections were treated with the plant growth hormones such as 2,4-dichlorophenoxyacetic acid (2,4-D) or indole-3-acetic acid (IAA) to accelerate growth and to loosen cell walls. If the sections were then treated with N-ethylmaleimide (NEM) or other thiol reagents, wall extensibility remained high, at the control level, whereas cell enlargement ceased (Fig. 1).

Solute leakage measured by conductivity changes did occur in the presence of NEM but the change after 1.5 h when growth inhibition was complete accounted for only a 5 to 6% reduction in internal osmotic pressure (Eisinger and Morré, 1968). Moreover, when sections first incubated in NEM were then transferred to a sulfhydyl protectant dithiothreitol (DTT), growth was restored with no effect on the rate of solute leakage. These experiments demonstrate that plant cell enlargement, at least, is not the result of turgor-drive expansion of auxin-loosened cell walls but occurs by a sulfhydryl reagent-blocked active mechanism.

There is no obligatory requirement for delivery of new membrane materials to the plasma membrane for cell enlargement to occur

Previous studies demonstrate that delivery of new membrane materials to the plasma membrane is not required for cell enlargement to occur (Morré 1994). One of these is that in response to temperature, elongating segments of soybean show extensive accumulations of membranes at the trans Golgi network at temperatures of 18°C or less. These accumulations are reminiscent of temperature blocks seen in other plant and animal cells (Tartakoff 1986). On the other hand, auxin-induced growth showed no sharp transition in response to temperature over the entire range of 4 to 25°C (Morré 1994). This would argue that elongation growth in plants induced by auxin occurs in a manner independent of the vesicular transport pathway.

Similar conclusions were reached earlier based on results

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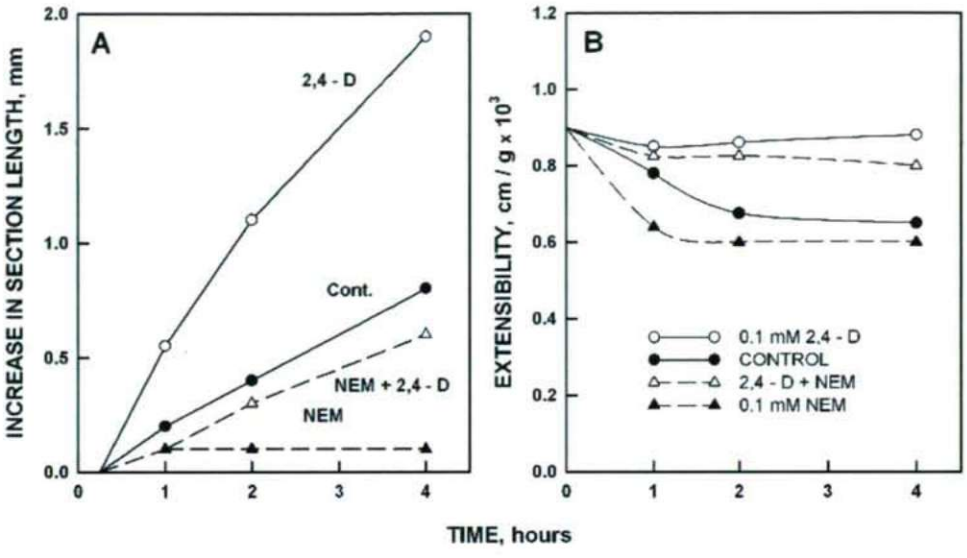

Figure 1. Time course of cell elongation (A) and extensibility (B) of 1 cm etiolated pea 3rd internode sections in the presence and absence of 0.1 mM 2,4-D with and without 0.1 mM N-ethylmaleimide (NEM). Redrawn from Eisinger and Morré (1968).

with monensin done with the narrow setae of the moss Pellia (Morré et al. 1986a). The setae elongate very rapidly and respond to auxin. Yet they are thin enough that the monensin is able to penetrate all of the cells in the section. That the monensin is able to penetrate is evidenced by electron microscope observations of swollen trans elements following fixation with glutaraldehyde (Morré et al. 1986b). Despite the nearly complete inhibition by monensin of normal trans Golgi apparatus functioning, treated setae responded in a relatively normal fashion to auxin for times of 4 h or more after the onset of monensin inhibition. Beyond 4 h, auxin-induced elongation ceased rather abruptly, possibly due membrane rupture as a result of the depletion of essential plasma membrane precursors required for sustained cell expansion.

ECTO-NOX proteins as drivers of cell enlargement

Growth-related ECTO-NOX proteins were first observed with isolated plasma membrane vesicles of soybean where activity was stimulated in response to the synthetic auxin 2,4 dichlorophenoxyacetic acid (2,4-D; Morré et al. 1986b) and inhibited by thiol reagents (Morré et al. 1995a). The acceptor was oxygen (Morré and Brightman 1991) or, in some cases, protein disulfides (Chueh et al. 1997).

Also extensively investigated, was a corresponding NADH oxidase activity of the mammalian plasma membrane (Brightman et al. 1992). The mammalian activity was both simulated by growth factors and hormones (Bruno et al. 1992) and inhibited by thiol reagents (Morré and Morré 1995). A strong correlation between cell enlargement and ECTO-NOX activity has since been demonstrated using both inhibitors and activators for plant as well as animal cells (Morré 1998a; Morré and Morré 2003).

Discussion

The marked susceptibility to inhibition by thiol reagents of both 2,4-D-induced growth and the 2,4-D-induced NADH oxidase of soybean plasma membrane suggested initially an involvement of essential active site thiols of the oxidase in the auxin growth mechanism. A similar thiol dependency was seen with the constitutively-activated NADH oxidase activity (CNOX) of hepatoma and HeLa cell plasma membranes.

If, indeed, the hormone- and growth-factor-stimulated NADH oxidase is in reality a thiol oxidoreductase or a thiol interchange protein, then the activity should exhibit, as well, a protein disulfide isomerase-like activity. Plasma membrane vesicles do exhibit protein-disulfide isomerase activity (Morré et al. 1995b). This activity with plant plasma membrane vesicles is stimulated approximately two-fold by auxin. While it is clear that long-term cell elongation extending over periods of several hours may be dependent upon the vesicular pathway of membrane addition for a source of membranes and membrane precursors, short term auxin-induced growth, that which occurs over an initial period of several hours, seems to occur more or less independently of a vesicular mechanism and independently of turgor as a driving force.

Based on the above findings, we have developed a model whereby cell enlargement is an active process involving both

ECTO-NOX proteins and an ATP-requiring step amenable to evaluation in a completely cell-free system with recombinant proteins and completely synthetic membrane vesicles. Findings support a central role of plasma membrane redox as an essential component of the cell enlargement process.

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Is Nox the source of ROS involved in Glut1 activity in B1647 cells?

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ABSTRACT The discovery of a family of superoxide-generating enzymes, homologues of phagocyte oxidase, has led to the concept that ROS are "intentionally" generated with biological functions in various cell types. We have recently shown that, in two leukaemic cell lines (M07e and B1647), there is a correlation between ROS and an important physiological activity, like glucose uptake, which is up-regulated in leukaemic cells. In this study, we tried to elucidate the sources of ROS generation and the mechanisms by which ROS are involved in the regulation of glucose uptake in B1647 cells. In particular, we investigated the presence and the role of a member of the NAD(P)H oxidase family (Nox). Data obtained in the presence of Nox inhibitors suggest that ROS involved in glucose uptake could be generated by this membrane-bound enzymatic complex. The effects of tyrosine kinase inhibitors and antioxidants show the importance of phosphorylation processes in the regulation of glucose uptake. PI3-kinase seems to be involved in ROS generation, possibly through Rac, which binds to Nox. The activation of tyrosine kinase receptor by vascular endothelium growth factor (VEGF), produced by an autocrine pathway in this cell line, seems to be an important step of this pathway.
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KEY WORDS

reactive oxygen species
glucose transport
NAD(P)H oxidase
leukaemic cell line

Reactive oxygen species (ROS), conventionally thought as cytotoxic and mutagenic, are now considered intracellular mediators of growth, senescence and apoptosis, when present at low level. The discovery of a family of superoxide-generating enzymes, homologues of phagocyte NAD(P)H oxidase (Nox2), has led to the concept that ROS are "intentionally" generated with biological functions in various cell types (Lambeth 2004).

We have recently shown (Fiorentini et al. 2004) that, in two leukaemic megakaryocytic cell lines (M07e and B1647), there is a correlation between ROS and an important physiological activity, like glucose uptake, which is up-regulated in leukaemic cells. Glut1, the transporter isoform present in these cell lines, is responsible for the basal glucose uptake in many cell types and is subjected to acute regulation by several metabolic and oxidative stresses.

In this study, we tried to elucidate the sources of ROS generation and the mechanisms by which ROS are involved in the regulation of glucose uptake in B1647 cell line, established from bone marrow cells of a patient with acute myelogenous leukaemia. In particular, we investigated the presence and the role of a member of the NAD(P)H oxidase family (Nox).

Materials and Methods

Chemicals

4-hydroxy-3-methoxyacetophenone (apocynin), capsaicin, diphenylene iodonium chloride (DPI), 2-deoxy-D-glucose (DOG) and phloretin, were purchased from Sigma Chemical (St. Louis, MO, USA). 2-Deoxy-D-[2,6-^{3}H]-glucose was from Amersham (UK); Iscove's modified Dulbecco's medium (IMDM) was purchased from Gibco (Grand Island, NY, USA); normal human serum (NHS) were from Hyclone (Holland). WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium) and PMS (1-methoxy-5-methylphenazinium methylsulfate) were purchased by Dojindo (Japan). All other chemicals were of the highest available purity grade.

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Cells and cell cultures

B1647 erythro-megakaryocytic cell line, established from bone marrow cells obtained from a patient with acute myelogenous leukaemia, is maintained in IMDM supplemented with 5% normal human serum (Bonsi 1997). These cells do not need growth factor addition for proliferation.

Cell viability evaluation

Total cell number was determined using a Bürker haemocytometer; the viable cells were evaluated by the Trypan blue exclusion test. In order to verify the absence of effect on cell viability and proliferation of tested inhibitors, MTT assay was used (Mosmann 1983).

Measurement of trans-plasma membrane electron transport (t-PMET)

t-PMET was measured by a simple dye reduction assay using

the cell-impermeable tetrazolium salt WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium) (Berridge and Tan 1998). In the presence of the intermediate electron acceptor PMS (1-methoxy-5-methylphenazinium methylsulfate), WST-1 is reduced to its soluble formazan at the cell surface via reducing equivalents derived from intracellular NAD(P)H, as described by Berridge (2005). Briefly, exponentially growing cells were washed twice, resuspended in PBS to a density of 10^6cells/mL and incubated with WST1/PMS solution (final concentration 500 μM WST-1, 20 μM PMS). Dye reduction was monitored as the absorbance at 450 nm – 580 nm in a dual wavelength Jasco V-555 UV/Vis spectrophotometer in the presence or absence of inhibitors (DPI, apocynin, capsaicin) preincubated for 20 min at 37°C.

Glucose transport assay

To evaluate glucose transport rate, cells (4 x 10^6/ml) were suspended in PBS and treated with a mixture of 2-deoxy-D-[2,6-^{3}H]glucose (0.5 μCi/assay) and 1.0 mM unlabeled glucose analogue for 1 min at 37°C under conditions where the uptake was linear at least for 10 min. After this time, the uptake was stopped by adding phloretin (final concentration 0.3 mM), a potent inhibitor of glucose transport. Cells were pelleted at 4,000 x g for 1 min and washed with PBS. Sample radioactivity was measured by liquid scintillation counting.

Statistical analysis

Each experiment was repeated three times with at least three parallel samples. Data are expressed as mean ± SEM. Statistical analysis was performed with Graph Pad Prism 4.

Results

Human phagocyte flavocytochrome b_{558} is an integral membrane protein composed of two polypeptides (gp91phox and gp22phox), that serves as the electron transferase of the NAD(P)H oxidase complex, generating superoxide anion when assembled with cytosolic subunit (p40phox, p47phox, p67phox and Rac). Homologues of human gp91phox have been identified in a variety of tissues. Recently, these novel proteins (Nox family) have been shown to play unique roles in development and signal transduction (Bokoch and Knaus 2003; Lambeth 2004). Progress in the study of Nox protein expression has been impeded because of paucity of immunological probes (Baniulis et al. 2005). Therefore, in order to evaluate the presence of a trans-plasma membrane electron transport (t-PMET) responsible of ROS generation in a leukaemic cell line, we used a simple dye reduction assay developed by Berridge and co-workers (1998). This method is based on the cell-impermeable tetrazolium salt (WST-1) that, in the presence of the intermediate electron acceptor PMS, is reduced to its soluble formazan at the cell surface via reducing equivalents derived from intracellular NADH.

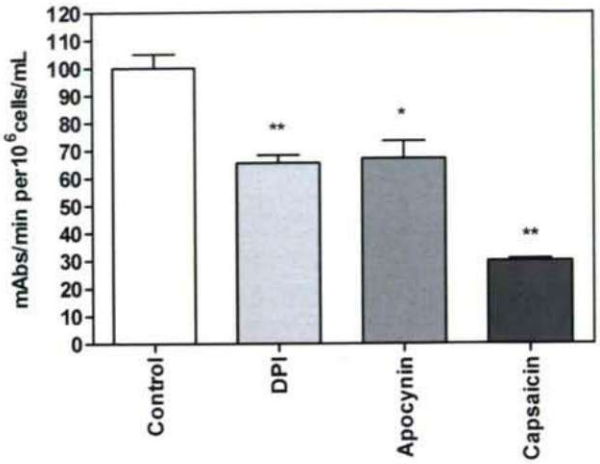

Figure 1. Effect of DPI, Apocynin and Capsaicin on t-PMET activity in B1647 cell line. Different from the control at level of significance of $p<0.05$ (*) and $p<0.01$ (**).

The physiological electron acceptor for t-PMET is oxygen (Scarlett et al. 2004).

By using inhibitors, such as, diphenylene iodonium, apocynin and capsaicin, we tried to investigate the structure of t-PMET in the B1647 cell line. In particular, diphenylene iodonium (DPI) is considered a non specific NAD(P)H oxidase inhibitor as it is capable of inhibiting additional flavin-dependent enzymes (Shen et al. 2006; Li and Trush 1998); apocynin (4-hydroxy-3-methoxy-acetophenone) is a specific NAD(P)H oxidase inhibitor that blocks the assembly of cytosolic subunit p47phox to the membrane complex (Dodd-O and Pearse 2000) and capsaicin, a homovanillic acid derivative (8-methyl-N-vanillyl-6-noneamide), a ubiquinone analogue (Herst et al. 2004), that inhibits CoQ redox cycling thought to be involved as electron shuttle in t-PMET (Scarlett et al. 2004). Treatment of B1647 cells with 10 μM DPI, 1 mM apocynin or 100 μM capsaicin for 20 min caused a significant decrease of WST-1/PMS reduction by 35%-40%-60%, respectively, as shown in Figure 1.

Since we demonstrated that there is a link between ROS generation and glucose uptake, in this leukaemic cell line (Fiorentini 2004; Prata 2004), we tested the effect of DPI, apocynin and capsaicin on glucose transport in order to investigate if t-PMET, a model of Nox, could be the ROS source involved in this process. Data reported in Figure 2 show that pretreatment of B1647 cells with the tested compounds resulted in a decreased glucose uptake by approximately 40%, 50% and 80%, respectively. The inhibitor effect of DPI, apocynin and capsaicin either on t-PMET or on glucose uptake revealed that this enzymatic complex seems to be crucial for ROS generation involved in glucose transport.

According to data reported in literature (Droge 2002; Chiarugi and Cirri 2003; Dusting et al. 2005), we hypoth-

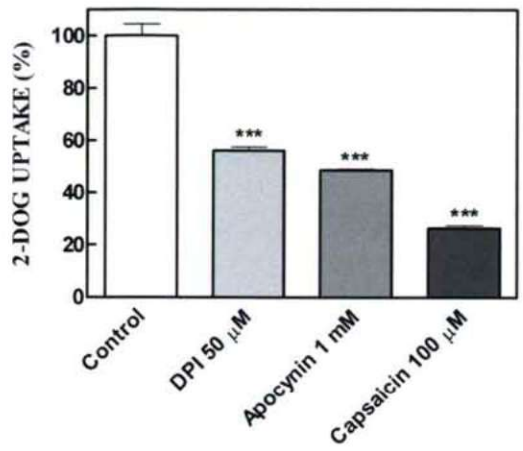

Figure 2. Effect of DPI, Apocynin and Capsaicin on glucose uptake, mediated by Glut1, in B1647 cell line. Different from the control at level of significance of $p<0.001$ (***).

esized that ROS could be generated by NOX after activation of tyrosine kinase receptors linked to growth factors. In this case the growth factor involved is VEGF, recently demonstrated to be produced by an autocrine pathway in B1647 cells (Bonsi et al. 2005). The small GTPase protein Rac1 seems to play a key role, since our preliminary results showed that Rac1 inhibitor (NSC23766) caused a significant decrease both in glucose uptake mediated by Glut1 and in intracellular ROS level. Moreover, since ROS may regulate activities of redox-sensitive enzymes, including protein phosphatases and, consequently, kinases (Chiarugi and Cirri 2003), by using antioxidants, we found that ROS are crucially involved in tyrosine phosphorylation, an important process that resulted important in the regulation of Glut1 activity in B1647 cells (not shown).

Discussion

Glucose transport regulation plays a key role in the aberrant growth of cancer cells, frequently characterized by high intracellular ROS level (Szatrowski and Nathan 1991; Toyokuni et al. 1995). Our previous results showed that ROS are involved in the modulation of Glut1 activity in B1647 cell line, where glucose uptake and basal level of intracellular ROS are higher than in normal cells (Fiorentini et al. 2004). Searching for the origin of ROS production, we focused on a family of membrane-localized NAD(P)H oxidase, Nox, homologus to the phagocytic one and present in various types of cells and tissues (Lambeth 2004).

The effects of DPI (a non specific NAD(P)H oxidase inhibitor), apocynin (a specific NAD(P)H oxidase inhibitor) and capsaicin (an ubiquinone analogue that inhibits CoQ redox cycling) on the trans-plasma membrane electron transport (t-PMET) revealed the involvement of flavoproteins, p47 phox subunit and CoQ cycle, respectively, in this NAD(P)H oxidase activity. Moreover, data obtained in the presence of the same inhibitors suggested that ROS involved in glucose uptake could be generated by this membrane-bound enzymatic complex. Data obtained in our laboratory (not shown) suggest a multistep pathway resulting in maintaining high Glut1 activity, in which phosphorylation process, modulated by ROS, plays a key role. In particular, PI3-kinase seems to be involved in ROS generation, possibly through Rac that, in turn, binds to NAD(P)H oxidase, as reported for other cell lines (Seshiah et al. 2002). An important step of this pathway should be also the activation of the tyrosine kinase receptor linked to vascular endothelium growth factor (VEGF), that is autoproduced in this cell line, as recently published (Bonsi et al. 2005). Moreover, VEGF has been reported to be induced by Nox1 in some tumor cells (Arbiser 2002); therefore, despite of the lack of specific Nox immunoprobe availability (Baniulis 2005), we can speculate that this isoform could be present in B1647 cell line. It's still remain unclear how ROS generated outside the cell penetrate inside and/or if different isoforms of Nox coexist in the same cell type, some of which producing ROS extracellularly and others intracellularly, as the simultaneous presence of multiple Nox proteins was demonstrated in one cell type, endothelial cells (Lassegue et al. 2001).

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Protein-protein interaction of cytochrome b_{561} in chromaffin vesicle membranes studied by two-dimensional blue-native/sodium dodecyl sulfate gel electrophoresis and co-immunoprecipitation analysis

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ABSTRACT We analyzed a protein-protein interaction in solubilized chromaffin vesicles using two-dimensional electrophoresis (1st, Blue-Native PAGE; 2nd, Tricine-SDS-PAGE). Cytochrome b_{561} band, which was verified by immunoblotting, was observed in the two-dimensional gel with an apparent molecular weight of ~100~400kDa. On the other hand, purified cytochrome b_{561} showed a monomeric band (28 kDa) in Blue-Native PAGE. These results indicated that cytochrome b_{561} interacts with other proteins in the chromaffin vesicles to form a large protein complex(es). To clarify the nature of the interaction, we performed co-immunoprecipitation experiments, where the solubilized membrane proteins were treated with immunopurified anti- b_{561} IgG antibodies followed by sedimentation with protein-A-Sepharose. We found that there were no other proteins co-sedimented with cytochrome b_{561}. Since the immunopurified anti- b_{561} IgG antibodies bound specifically to the C-terminal hydrophilic portion of cytochrome b_{561} protein, we concluded that such binding of the IgG antibodies to the C-terminal portion might cause an inhibition of protein-protein interaction with other proteins in the solubilized state.
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KEY WORDS

Cytochrome b_{561} protein-protein interaction
Blue-Native PAGE
two-dimensional gel electrophoresis
chromaffin vesicles
co-immunoprecipitation

In the matrix and membranes of chromaffin vesicles, there are many proteins, peptides and enzymes responsible for various physiological functions (Winkler et al. 1986). In the vesicular matrix, three major secretory proteins (chromogranin A and secretogranins I and II) together with peptides derived from them, and smaller amounts of neuropeptides, and several different endo- and exo-proteinase for the production of these peptides exist (Apps 1997). In the membranes, those responsible for catecholamine biosynthesis (dopamine β-hydroxylase and cytochrome b_{561}), active transport of vesicle components (V-type ATPase, carriers for monoamines, nucleotides, and small ions) and exocytosis (synaptotagmin, synaptobrevin and other proteins) are known to exist. To perform these specific biological roles, these proteins and other proteins with unknown functions must have many kinds of interactions each other and, in sometimes, form stable or transient molecular complexes. Such formations of the protein complexes are usually crucial to manifest most of their physiological functions.

Among these proteins residing in chromaffin vesicles, cytochrome b_{561} is very unique to have a role for transporting electrons from cytosolic ascorbate (AsA) to intravesicular monodehydroascorbate (MDA) radical to regenerate AsA (Njus and Kelley 1993). This electron transfer is essential for the production of neurotransmitters by intravesicular copper-containing monooxygenases, dopamine β-hydroxylase (DBH) and peptidylglycine α-amidating monooxygenase (PAM) (Apps 1997; Winkler and Fischer-Colbrie 1998). It is assumed that cytochrome b_{561} exists in the membrane as a monomer and plays a role as an electron conduit from cytosolic AsA to intravesicular MDA radical. However, there have been some reports describing a small intravesicular protein, which may interact with cytochrome b_{561} and participate in some role for the electron transfer or its modulation (Grigoryan et al. 1981; Markossian et al. 1986). Further, a direct interaction between cytochrome b_{561} and the membrane-bound form of DBH was inferred (Ahn and Klinman 1987; Wimalasena and Wimalasena 1995). In the present study, we examined such possibilities of a direct protein-protein interaction of cytochrome b_{561} with other proteins in chromaffin vesicles using a various biochemical techniques, including two-dimensional Blue-Native PAGE, co-immunoprecipitation, and immunoblotting. Blue-Native electrophoresis was originally developed to study mitochondrial membrane protein complexes (Schägger and von Jagow 1991; Schägger et al. 1994; Schägger and Pfeiffer 2000) and has since then been used widely (van Lis et al. 2003; Culvenor et al. 2004; Claeys et al. 2005). Us-

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ing this technique, membrane proteins and their complexes were solubilized with a nonionic detergent and native protein-protein complexes were resolved in a first-dimensional PAGE in the presence of Coomassie Brilliant Blue (CBB) G250 dye according to their apparent molecular mass. In a second dimension, an SDS-PAGE was employed, where SDS denatured the complex and separates them into their respective subunits according to their molecular mass during electrophoresis. A preliminary study on chromaffin vesicle membrane proteins with this technique was reported previously by Apps (Apps 1997), in which a single protein-staining band corresponding to cytochrome b_{561} was observed together with DBH (monomer and tetramer) and V-type ATPase (115-,39- and 16-kDa subunits of the V_o segment). However, since then, there has been no detailed study concerning such protein-protein interactions in chromaffin vesicle membranes. In the present study, we found that cytochrome b_{561} is likely to have protein-protein interactions with other proteins and that the C-terminal hydrophilic tail may have some roles for such interactions.

Materials and Methods

Purification of anti-cytochrome b_{561} IgG

Antisera against purified bovine cytochrome b_{561} (Tsubaki et al. 1997) were raised in a Japanese white rabbit (Takara-Bio, Japan) and were partially purified by ammonium sulfate fractionation (0~0.2M). The partially purified IgG fraction was further purified at 4°C using HiTrap-NHS- b_{561} affinity column, in which purified bovine cytochrome b_{561} was covalently attached to HiTrap-NHS activated HP resin (1.0 mL volume pre-packed in a column; Amersham Biosciences, USA) according to the manufacturers recommendation. The adsorbed anti-cytochrome b_{561} IgG was washed with 1M NaCl, 1% Triton X-100, 20 mM Tris-HCl (pH 7.5) at 4°C and, then, eluted with 0.1 M Gly-HCl pH 2.5. The eluted IgG fraction was neutralized immediately by mixing with 1 M Tris and was further mixed with 1.0 mg/ml of bovine serum albumin to increase the stability.

Sample preparation for blue-native electrophoresis

Chromaffin vesicles were obtained from bovine adrenal medullae as previously described (Tsubaki et al. 1997). The purified vesicles were saved in –80°C until use. To solubilize chromaffin membrane proteins and their complexes, the membrane pellet was vigorously pipetted in extraction buffer (final volume of 100 µl with 4.0 mg protein/ ml) containing 750 mM aminocaproic acid, 50 mM BisTris/HCl (pH 7.0) at 4°C. Fifteen µl of 10% (w/v) dodecyl maltoside were then added to the suspension (final 0.34% (w/v)). After incubation on ice for 20 min with vortex mixing in every 5 minutes, insoluble membrane materials were removed by centrifugation at 10,500 rpm for 20 min. To the supernatant (155 µl), 2.5 µl of 5% (w/v) Coomassie Brilliant Blue G250 diluted in 500 mM aminocaproic acid was added. Samples were then briefly centrifuged at 10,500 rpm to eliminate any precipitate and kept on ice until loading on Blue-Native (BN) gel.

Blue-Native PAGE and immuno blot analyses

Blue-Native (BN)-PAGE for soluble and membrane proteins was carried out according to a published protocol (Schägger and von Jagow 1991; Schägger et al. 1994) using 5-18% gradient gel. The native high-molecular-weight markers (66~669 kDa) used for calibration were as follows; thyroglobulin (669 kDa), ferritin (440 kDa), catalase (250 kDa), and bovine serum albumin (66 kDa) (from Sigma, USA). Additionally, purified cytochrome *c* oxidase (from bovine heart mitochondria, 220 kDa (dimer)) and dopamine β-hydroxylase (from bovine adrenal medulla (350 kDa (tetramer)) were used. One complete lane was excised from the fist dimensional, and the gel was resolved by a denaturing Tris-SDS-PAGE in the second dimension, after an appropriate pre-treatment. After the electrophoresis, proteins in the gels were transferred to a nitrocellulose membrane (0.45 µm, BioRad, USA) by semidry blotting by using a discontinuous transfer system in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol with 150 mA for 1.5 h. Blocking and immunodecorations were performed in Tween-20/phosphate-buffered saline containing 1% (w/v) BSA using anti-bovine cytochrome b_{561}-IgG(rabbit) (500-fold dilution) as primary antibodies and anti-rabbit IgG-IgG(goat)-HRP conjugate (500-fold dilution) as secondary antibodies. Immunodetection was done by addition of 0.2% 4-chloro-1-napthol in 50 mM Tris-HCl (pH7.4) followed by 30% hydrogen peroxide (final 0.006%).

Co-immunoprecipitation analyses

Bovine chromaffin vesicle membranes were solubilized with 1.0% (w/v) dodecyl maltoside in the same buffer used for Blue-Native PAGE. After the centrifugation (10,500 rpm, 10 min) to remove insoluble materials, the solution was incubated with purified anti-cytochrome b_{561} IgG (rabbit) using a rotary incubator (30min) followed by precipitation with protein-A-Sepharose beads (centrifugation at 2,000 rpm; 2 min). The precipitate was washed with 0.1% (w/v) Triton X-100 in 0.15 M NaCl and 50 mM Tris-HCl (pH 7.4) to remove non-specifically bound proteins. Then, the precipitate was boiled to release proteins from the bound complex. Then, the released proteins were analyzed by Tricine-SDS-PAGE (Schägger and von Jagow 1987) in the absence of disulfide-bond reducing reagents (dithiothreitol or β-mercaptoethanol). As control experiments, non-specifically interacting proteins, either in the solubilized chromaffin vesicles or in the purified IgG fraction, to protein-A-Sepharose beads were analyzed in the same condition.

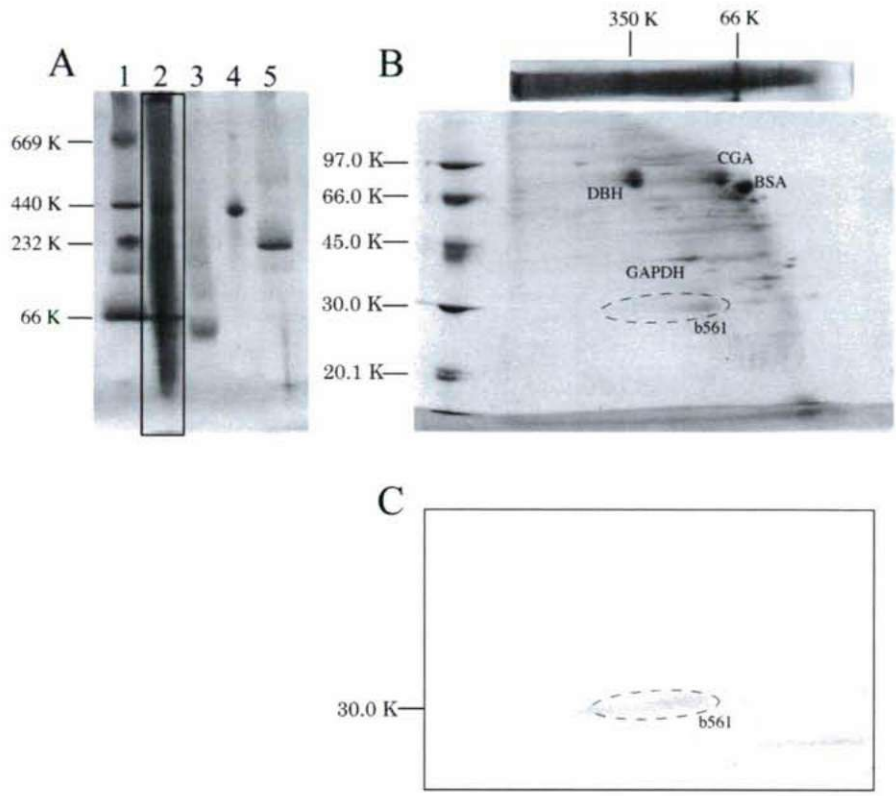

Figure 1. One-dimensional and two-dimensional resolution of bovine chromaffin vesicle membrane proteins using Blue-Native PAGE. (Panel A) Bovine adrenal chromaffine vesicle membranes were solubilized, and resolved by Blue-Native PAGE in first native dimension (lane 2). Purified bovine cytochrome b_{561} (12.9 μg, lane 3), bovine dopamine β-hydroxylase (lane 4), bovine cytochrome *c* oxidase (lane 5), and a mixture of protein markers (lane 1: thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; bovine serum albumin, 66 kDa) were also electrophoresed under the same condition. Gel concentration, 5~18% linear gradient; 0.085% CBB G-250; 0.34% dodecyl maltoside. (Panel B) One complete lane (lane 2) was excised from the fist dimensional gel (indicated by a rectangle) was resolved by a denaturing Tris-SDS-PAGE in the second dimension, after an appropriate pre-treatment. In the far-left of the gel, a mixture of low-molecular weight markers (97.0 kDa, 66.0 kDa, 45.0 kDa, 30.0 kDa, 20.1 kDa, 14.4 kDa; LMW Marker Kit, Amersham Biosciences, USA) was applied. The gels were stained with Coomassie Brilliant Blue R-250. Cytochrome b_{561} protein spot was circled for easiness of the identification. Other identified proteins (DBH, BSA, CGA, and GAPDH) were also indicated. (Panel C) The resolved proteins in the gel after the second dimensional SDS-PAGE were transferred to a nitrocellulose membrane and cytochrome b_{561} protein was visualized using immunochemical technique as described in the text and was circled for easiness of the identification.

NH_2-terminal amino acid sequencing of protein spots in 2D gel

NH_2-terminal amino acid sequence of each protein spot in 2D gel was analyzed as follows. Proteins in the 2D gel were electro-blotted onto a PVDF membrane (Sequi-Blot, 0.2 μm; BioRad, USA). The Ponceau S-stained protein band on the PVDF membrane was cut using a clean razor and was directly analyzed with an ABI protein sequencer (Model 492; Applied Biosystems, USA) up to 10 cycles.

Results and Discussion

Blue-Native PAGE analyses of chromaffin vesicle membrane proteins and cytochrome b_{561}

The solubilized chromaffin vesicle membranes were analyzed by Blue-Native PAGE with 5~18% linear gradient polyacrylamide gel (Fig. 1A, lane 2). It showed several proteins or protein complexes with apparent molecular weights at ~30 kDa, 66 kDa and 350 kDa. These protein bands were overlapped with several broad bands centered 50~500 kDa. To analyze these overlapping protein bands in the first dimension, the rectangular section in Fig. 1A was excised and was resolved further in a denatured condition by Tricine-SDS-PAGE. A representative 2D gel stained with CBB R-250 after the electrophoresis is shown in Fig. 1B. The 350 kDa and 66 kDa band in the first Blue-Native PAGE were found as a tetrameric form of DBH (87.5 kDa) and a monomeric form of BSA (66 kDa). In addition, protein bands for chromogranin-A precursor (CGA) (50 kDa) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (36 kDa) were identified. The identification of these 4 protein bands was based on the NH_2-terminal amino acid sequences up to 10 cycles on the

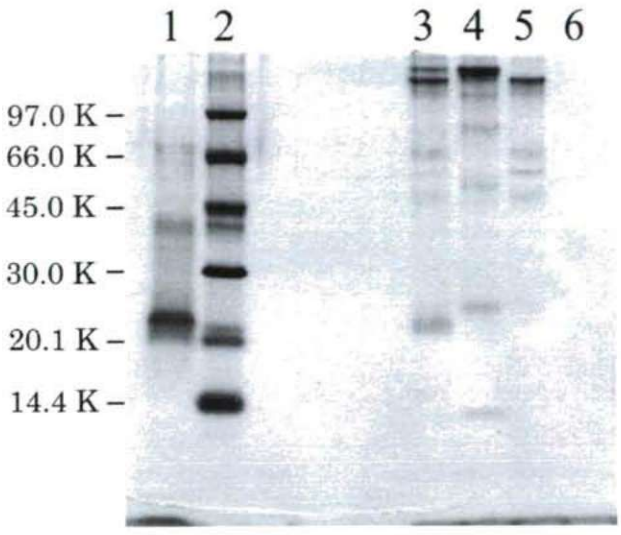

Figure 2. Immunoprecipitation of bovine cytochrome b_{561} using purified anti- b_{561} IgG and its interaction with other proteins analyzed by SDS-PAGE. Bovine chromaffin vesicle membranes were solubilized with 1.0% (w/v) dodecyl maltoside and the solution was incubated with purified anti-cytochrome b_{561} followed by precipitation with protein-A-Sepharose beads. Then the precipitate was boiled and released proteins were analyzed (lane 3), showing a band of cytochrome b_{561} and other non-specifically bound proteins with a higher molecular weight. As controls, non-specifically bound proteins in chromaffin vesicles (lane 4) and non-specifically bound proteins in purified IgG fraction (lane 5) to protein-A-Sepharose beads are shown. There was no release of proteins from protein-A-Sepahrose beads after boiling (lane 6).

transferred proteins to PVDF membranes from the gel and MALDI-TOF analyses for in-gel-digested tryptic peptides. The CGA showed an apparent molecular weight of ~100 kDa whereas GAPDH indicated a mobility of about 250 kDa, both in the Blue-Native gel. These results suggested that the formation of a stable molecular complex with other proteins for CGA (Yoo et al. 2005) and a dimer of tetrameric complex for GAPDH in native conditions.

Cytochrome b_{561} in the solubilized chromaffin vesicle membranes did not form a sharp protein spot in the 2D gel (Fig. 1B). Instead, it formed a very broad and spread protein band ranging from 100 kDa to 400 kDa in the first dimension and 28kDa in second dimension. This broad band as an authentic cytochrome b_{561} protein was confirmed by immunodetection using anti-cytochrome b_{561} IgG(rabbit) and anti-rabbit IgG-IgG(goat)-HRP conjugate (Fig. 1C). However, when the purified bovine cytochrome b_{561} was analyzed in the native form under the same buffer and detergent condition, it showed a clear protein spot (~30kDa) in the first Blue-Native PAGE gel (Fig. 1A, lane 3). These results suggested that cytochrome b_{561} is likely to form a large protein complex with other membrane (or soluble) proteins in the solubilized chromaffin vesicle membranes. Formation of a homo-oligomeric forms of cytochrome b_{561} might be not so significant, since the formation of such complexes were not so obvious for the purified cytochrome b_{561} in the native form (Fig. 1A, lane 3). To clarify these interacting proteins with cytochrome b_{561} in chromaffin vesicles, we conducted co-immunoprecipitation analyses.

Specificity of anti-b_{561}-IgG antibodies

Before the immunoprecipitation analyses, we investigated a binding specificity of the immunopurified anti- b_{561}-IgG antibodies to the purified bovine cytochrome b_{561} by Western blotting. When a partially digested mixture (with endogenous proteases) of cytochrome b_{561} was analyzed, the immunopurified anti- b_{561} IgG antibodies recognized the full-length of cytochrome b_{561} protein. However, the immunopurified anti-b_{561} IgG antibodies did not recognize the partially digested form in which only the C-terminal portion was specifically cleaved off (not shown). Therefore, we concluded that the immunopurified anti- b_{561} IgG antibodies recognized specifically the C-terminal hydrophobic peptide. Being consistent with this conclusion, when a purified fused protein DHFR-Bb561C, in which a C-terminal part (219-252) of bovine cytochrome b_{561} was fused to the C-terminus of DHFR and was expressed in *E. coli*, was analyzed similarly, the immunopurified anti- b_{561} IgG antibodies recognized this protein very clearly (not shown).

Co-immunoprecipitation analyses

Immunopurified anti-b_{561}-IgG antibodies were mixed with the solubilized chromaffin vesicle membranes to form putative (anti-b_{561}-IgG)-(b_{561})-(interacting protein) ternary complexes in the solution. Such putative protein complexes were sedimented by mixing with protein-A-Sepharose beads, washed, and followed by boiling to release the individual protein molecules from the complex. The released proteins were then analyzed with Tricine-SDS-PAGE (Schägger and von Jagow 1987), but in the absence of disulfide-bond reducing reagents (dithiothreitol or mercaptoethanol). A protein band corresponding to cytochrome b_{561} (28 kDa) and other faint protein bands with a higher molecular weight were observed (Fig. 2, lane 3). As control experiments, non-specifically bound proteins in chromaffin vesicles (Fig. 2, lane 4) and non-specifically bound proteins in purified IgG fraction (Fig. 2, lane 5) to protein-A-Sepharose beads were also analyzed. There was no release of proteins from protein-A-Sepharose beads after boiling (Fig. 2, lane 6). Since all the protein bands with a higher molecular weight appeared in lane 3 were identified as non-specifically bound proteins to protein-A-Sepharose beads (lanes 4 and 5), we reached a conclusion that there was no protein having a direct interaction with cytochrome b_{561} in the solubilized vesicle membranes, at least in the present experimental condition.

Protein-protein interacting domain of cytochrome b_{561}

To explain the present discrepant results, we thought a possibility that binding of purified anti- b_{561} IgG antibodies to the C-terminal part of cytochrome b_{561} protein may inhibit the interaction with other proteins. Since cytochrome b_{561} does not contain a cleavable signal peptide, it is predicted that both N-terminus and C-terminus would be localized to the cytoplasmic side of the secretory vesicle membrane (Perin et al. 1988). According to our membrane-spanning model of cytochrome b_{561} (Okuyama et al. 1998), most of the hydrophilic sequences of cytochrome b_{561} are on the cytoplasmic face of the vesicles, as has been suggested from biochemical experiments (Abbs and Phillips 1980). Indeed, we have shown that some parts of these cytosolic hydrophilic segments containing negatively-charged amino acid residues are responsible for the interaction with ascorbate (Tsubaki et al. 2000; Takeuchi et al. 2001) and the N-terminal Met residue is acetylated (Nakamura et al. 2003). The remaining C-terminal hydrophilic peptide (34 amino acid residue-long) is the longest part in cytochrome b_{561} exposed to the cytosolic surface but its physiological roles are still not well-understood. The most likely story is that this segment contains multiple routing signals (such as a targeting signal for the transit from Golgi to chromaffin vesicles and/or a retrieving signal from the plasma membrane to *trans* Golgi network), as has been identified for peptidiylglycine α-hydroxylating monooxygenase (Milgram et al. 1996). Experiments are currently in progress to identify proteins that may interact with this segment.

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The manufacturer's name and location should be given in parentheses for reagents and instruments. Sources for all antibodies and nucleotide sequences should be indicated. Customary abbreviations in common use need not be defined in the text (e.g. DNA or ATP). Other abbreviations should be defined the first time that they are used. Quantitative results must be presented as graphs or tables and supported by appropriate experimental design and statistical tests. Only SI units may be used. For studies that involve animals or human subjects, the institutional, national or international guidelines that were followed should be indicated.

References

Only work that has been published or is in the press may be referred to. Personal communications should be acknowledged in the text and accompanied by written permission. In the text, references should be cited by name and year, e.g. Bloom (1983) or (Schwarz-Sommer et al. 1990) or (Maxam and Gilbert 1977). In the References, references should be listed alphabetically by first authors (including all co-authors) and chronologically for a given author (beginning with the most recent date of publication). Where the same author has more than one publication in a year, lower case letters should be used (e.g. 1999a, 1999b, etc.). Periods should not be used after authors' initials or abbreviated journal titles (e.g. Acta Biologica Szegediensis should be cited as Acta Biol Szeged). Inclusive page numbers should be used. Examples:

Bloom FE (1983) The endorphins: a growing family of pharmacologically pertinent peptides. Annu Rev Pharmacol Toxicol 23:151-170.

Coons AH (1978) Fluorescent antibody methods. In Danielli JF, ed., General Cytochemical Methods. Academic Press, New York, 399-422.

Maxam AM, Gilbert WA (1977) A new method for sequencing DNA. Proc Natl Acad Sci USA 74:560-564.

Monod J, Changeux J-P, Jacob F (1963) Allosteric proteins and cellular control systems. J Mol Biol 6:306-329.

Schwarz-Sommer Z, Huijser P, Nacken W, Saedler H, Sommer H (1990) Genetic control of flower development by homeotic genes in Antirrhinum majus. Science 250:931-936.

Illustrations

Three complete sets, including a high-quality "original" for publication, must be submitted with the manuscript. The back of each figure or composite plate should be labeled in soft lead pencil, indicating the orientation, the figure number, and the first author's name. The back of the best set should be marked "use for reproduction" or "original". Authors are encouraged to submit digital images of photographs, line drawings or graphs for printing. Most major image editing and drawing/illustrator computer software files (both Macintosh and PC) in TIFF or EPS formats are acceptable. It is particularly important that adequate resolution (at least 300 dpi, preferably 600 dpi) is used in making the original image.

Figure legends

Figures should be numbered consecutively with Arabic numerals. Material in the text should not be duplicated and methods should not be described. The size of scale bars should be indicated when appropriate. The first figure in the text should be referred to as Fig. 1, and so on.

Tables

Tables should be numbered consecutively with Arabic numerals. A brief title should be included above the table. Each table should be printed double spaced, without vertical or horizontal lines, and on a separate sheet. Material in text should not be duplicated and methods should not be described. The first table in the text should be referred to as Table 1, and so on.